



eCOMMONS

Loyola University Chicago  
**Loyola eCommons**

---

Master's Theses

Theses and Dissertations

---

2010

# Localization and Functional Analysis of Plasmodium Falciparum Genes PFL2550w and PFF0750w

Carolyn Jane Strobel  
*Loyola University Chicago*

---

## Recommended Citation

Strobel, Carolyn Jane, "Localization and Functional Analysis of Plasmodium Falciparum Genes PFL2550w and PFF0750w" (2010).  
*Master's Theses*. Paper 491.  
[http://ecommons.luc.edu/luc\\_theses/491](http://ecommons.luc.edu/luc_theses/491)

This Thesis is brought to you for free and open access by the Theses and Dissertations at Loyola eCommons. It has been accepted for inclusion in Master's Theses by an authorized administrator of Loyola eCommons. For more information, please contact [ecommons@luc.edu](mailto:ecommons@luc.edu).



This work is licensed under a [Creative Commons Attribution-Noncommercial-No Derivative Works 3.0 License](https://creativecommons.org/licenses/by-nc-nd/3.0/).  
Copyright © 2010 Carolyn Jane Strobel

LOYOLA UNIVERSITY CHICAGO

LOCALIZATION AND FUNCTIONAL ANALYSIS  
OF PLASMODIUM FALCIPARUM GENES

PFL2550W AND PFF0750W

A THESIS SUBMITTED TO  
THE FACULTY OF THE GRADUATE SCHOOL  
IN CANDIDACY FOR THE DEGREE OF  
MASTER OF SCIENCE

PROGRAM IN BIOLOGY

BY

CAROLYN J. STROBEL

CHICAGO, IL

AUGUST 2010

Copyright by Carolyn J. Strobel, 2010  
All rights reserved.

## **ACKNOWLEDGEMENTS**

I would like to thank my thesis advisor, Dr. Kim Williamson, for all her guidance and support in my research project and other work in the lab. Her knowledge and expertise in this field enabled me to learn so much about malaria, and her guidance and instruction truly helped me to grow as a scientist. I would also like to thank my committee members, Dr. Stefan Kanzok and Dr. Catherine Putonti, for their help and encouragement during my time at Loyola. I would like to thank Dr. Terry Grande and Dr. Martin Berg for their leadership of the M.S. Biology graduate program. Thank you to all the wonderful members of the Williamson lab for being so helpful in the lab and for making my graduate school experience fun and memorable. A special thanks to Dr. Belinda Morahan, Dr. Uzma Hasan, Samrawit Goshu, and the students from Dr. Jutta Heller's BIOL 390 class in the fall semester of 2007 for their preliminary work on the genes and proteins used in my thesis research. Thank you to Dr. C. Braun Breton for the SBP1 antibodies and to Dr. Brendan Crabb for the HSP101 antibodies used in my research. Thanks to my fellow graduate students for their friendship and support over these two years. Lastly, thanks to my parents, sisters, and my husband, Jeremy, for their love and encouragement.

## TABLE OF CONTENTS

|   |      |
|---|------|
| ACKNOWLEDGEMENTS .....  | iii  |
| LIST OF FIGURES .....   | vi   |
| LIST OF TABLES .....  | viii |
| CHAPTER I: INTRODUCTION.....  | 1    |
| Proposal.....   | 1    |
| Literature Review.....  | 3    |
| CHAPTER II: MATERIALS AND METHODS.....                                  | 23   |
| Experimental Design.....  | 23   |
| DNA Amplification .....   | 24   |
| Bacterial Vectors.....  | 26   |
| Digestion .....   | 26   |
| Agarose Gels.....   | 27   |
| Ligation.....   | 28   |
| Transformation of <i>E. coli</i> .....                                  | 28   |
| Colony PCR .....  | 30   |
| Plasmid Isolation.....  | 31   |
| Restriction Analysis .....  | 31   |
| DNA Sequencing .....  | 32   |
| Plasmid DNA Amplification.....  | 32   |
| Parasite Transfection.....  | 32   |
| Integration PCR .....   | 35   |
| Limited Dilution Cloning.....   | 35   |
| Protein Construct Preparation .....                                     | 36   |
| Protein Expression .....  | 37   |
| Protein Purification .....  | 37   |
| Estimation of Protein Concentration.....                                | 39   |
| Mice Immunizations and Tail Bleeds .....                                | 39   |
| Isolation and Extraction of Parasites from Blood Culture .....          | 40   |
| Characterization of Antiserum.....                                      | 42   |
| Immunoprecipitation.....  | 44   |
| CHAPTER III: RESULTS.....   | 46   |
| Purification and SDS-PAGE Analysis of PFL2550w Recombinant Protein..... | 46   |
| PFL2550w Western Blot with Asexual and Gametocyte Extracts .....        | 46   |
| PFL2550w Western Blots with Saponin-treated Gametocyte Extract.....     | 48   |
| PFL2550w Western Blot with Timecourse Parasite Extracts.....            | 52   |
| PFL2550w Immunofluorescence Assays.....                                 | 55   |
| Colocalization IFA of PFL2550w and Pfs16.....                           | 57   |
| Counts of PFL2550w and Pfs16 Expressing Parasites .....                 | 57   |

|  |     |
|--|-----|
| Localization IFA with SBP1 .....   | 59  |
| PFL2550w Immunoprecipitation .....   | 62  |
| Colocalization IFA of PFL2550w and HSP101 .....                              | 66  |
| DNA Construct Preparation for Parasite Transfections .....                   | 69  |
| Transfection of PFF0750w Truncation Construct DNA into Parasites .....       | 71  |
| Purification and SDS-PAGE Analysis of PFF0750w Recombinant Protein .....     | 73  |
| PFF0750w Western Blot with Gametocyte and Gamete Extracts .....              | 74  |
| PFF0750w Immunofluorescence Assays .....                                     | 78  |
| CHAPTER IV: DISCUSSION .....   | 82  |
| PFL2550w .....   | 82  |
| Generation of PFL2550w-specific antibodies .....                             | 82  |
| Early gametocyte-specific expression of PFL2550w .....                       | 83  |
| Localization of PFL2550w to the host erythrocyte cytosol .....               | 86  |
| Interaction of PFL2550w with export-related and RBC-localized proteins ..... | 89  |
| Potential PFL2550w function .....  | 93  |
| PFL2550w summary .....   | 97  |
| PFF0750w .....   | 97  |
| Generation of PFF0750w-specific antibodies .....                             | 97  |
| Targeted PFF0750w gene disruption .....                                      | 100 |
| PFF0750w summary .....   | 101 |
| REFERENCES .....   | 102 |
| VITA .....   | 107 |

## LIST OF FIGURES

| Figure   | Page |
|--|------|
| 1. <i>Plasmodium falciparum</i> life cycle and five gametocyte developmental stages.....                                       | 5    |
| 2. Expression profile of PFL2550w throughout the asexual and sexual <i>P. falciparum</i> blood stages.....                     | 11   |
| 3. Expression profile of PFF0750w throughout the asexual and sexual <i>P. falciparum</i> blood stages.....                     | 17   |
| 4. Expression profiles of PFF0750w and PFI1710w throughout the asexual and sexual <i>P. falciparum</i> blood stages.....       | 18   |
| 5. Amylose resin purification of PFL2550w recombinant protein .....  | 47   |
| 6. PFL2550w western blot of asexual and gametocyte extract, with and without BME...  | 49   |
| 7. PFL2550w western blot of extracts from gametocytes treated without and with saponin (+sap).....                             | 51   |
| 8. PFL2550w western blot of extracts from parasite timecourse.....   | 54   |
| 9. IFA analysis of PFL2550w protein expression and localization throughout parasite development.....                           | 56   |
| 10. Colocalization IFA analysis of PFL2550w and Pfs16 antibodies on stage I and II gametocytes.....                            | 58   |
| 11. Giemsa-stained smear of Accudenz purified schizonts (S) and “non-schizonts” (NS) – trophozoites and early gametocytes..... | 60   |
| 12. Counts of total parasites, PFL2550w positive parasites, and Pfs16 positive parasites per 800 red blood cells.....          | 61   |
| 13. Western blot of PFL2550w immunoprecipitation.....  | 63   |
| 14. Expression profiles of immunoprecipitation-identified genes, PF11_0175 and PFE0040c, with PFL2550w.....                    | 67   |

|   |    |
|---|----|
| 15. Colocalization IFA analysis of PFL2550w and HSP101 antibodies on mixed stage gametocytes..... | 68 |
| 16. Restriction digest analysis of PFL2550w construct DNA for transfections.....                  | 70 |
| 17. Integration PCR analysis of PFF0750w gene truncation transfectants J1 and J2.....             | 72 |
| 18. Amylose resin purification of PFF0750w recombinant protein (E4) .....                         | 75 |
| 19. PFF0750w western blots of extracts from gametocytes and gametes .....                         | 77 |
| 20. IFA analysis of PFF0750w protein expression in <i>P. falciparum</i> gametes.....              | 80 |



## LIST OF TABLES

| Table  | Page |
|--|------|
| 1. Immunoprecipitation-identified proteins exclusive to PFL2550w column eluates..... | 65   |

## CHAPTER I

### INTRODUCTION

#### Proposal

Malaria is a parasitic disease that causes over a million deaths worldwide each year. *Plasmodium falciparum* causes the most severe form of malaria and is responsible for human fatalities. The parasite's complex life cycle consists of multiple phases in both its mosquito and human hosts. Gaining an understanding of the parasite's development through these various stages is necessary to develop new methods to stop the progression and transmission of *P. falciparum* malaria. As the genetic and molecular basis for the crucial transition from the asexual to the sexual blood stages in the human host is unknown, we hope to further understand this transition by studying sexual stage genes and their roles in gametocytogenesis.

PFL2550w and PFF0750w are two *P. falciparum* genes that are known to be expressed in the sexual stage, as the parasites progress through gametocyte production. Parasites must enter the sexual cycle to become gametocytes, which once taken up by a mosquito become gametes. The gametes then fertilize and differentiate into sporozoites that can be transmitted to another human host. Blocking this transition to gametocytogenesis or altering the development or function of gametocytes could prevent the production of mature stage V gametocytes, thus halting malaria transmission.

From previous microarray data (Young et al. 2005; Eksi et al. 2005; unpublished data Eksi et al.), the mRNA expression profiles of many sexual stage genes have been determined. From those genes, PFL2550w was selected for further study because it was found to be expressed at much higher levels in a parasite strain that produced gametocytes at high levels compared to a strain of gametocyte-deficient parasites (unpublished data Eksi et al.). The PFL2550w protein is known to contain a secretory signal sequence, an erythrocyte export domain, and a DnaJ domain, suggesting a potential role in the parasite's interaction with its host red blood cell (www.PlasmoDB.org [updated 2010]).

PFF0750w was selected for further analysis because its mRNA expression pattern is very similar to the expression pattern of PFI1710w, named Pfgdv1, a gene which was found to be deleted in a cultured strain of gametocyte-deficient parasites (unpublished data Eksi et al.). Additionally, as the PFF0750w protein is a predicted cyclin-dependent kinase, it may play an important role in regulating the parasites' transcription and in cell cycle regulation (PlasmoDB [updated 2010]). Separately, the genes will be studied by targeted gene disruption. To do this, a plasmid designed to integrate into the genomic copy of the gene will be generated and used to transform *P. falciparum* parasites in culture. After cloning the transfected parasites, the phenotype of the gene-truncated parasites can be determined. Additionally, recombinant proteins corresponding to both genes have been produced and purified. These recombinant proteins will be separately injected into mice for antibody production. The collected mouse antiserum will be used for western blots and immunofluorescence assays (IFAs) to determine protein localization and potential protein function in the malaria parasites.

Both genes potentially play important roles in malaria transmission and pathogenesis. Due to their increased mRNA expression during sexual differentiation and thus possible role in the process of gametocytogenesis, either gene may be found to be a potential vaccine or drug target candidate and antibodies against their proteins could have an important role in malaria transmission prevention. This research study hopes to determine that crucial role if it exists, or to eliminate these genes as drug target candidates if found to be insignificant to the parasites' life cycle. The overall goal of this study is to further analyze these two genes and their respective proteins to determine their specific functions in the life cycle of *P. falciparum* parasites.

### Literature Review

Malaria is a devastating tropical disease that is estimated to cause approximately one million deaths each year, primarily among African children (Kelly-Hope and McKenzie 2009). About 50% of the world's population lives in at-risk areas where malaria is transmitted by its insect host, the *Anopheles* mosquito (www.WHO.int [updated 2009]). Symptoms of malaria vary depending on whether the disease is classified as uncomplicated or severe. Symptoms of uncomplicated malaria typically include fever, chills, aches, nausea, and general malaise (www.CDC.gov [updated 2006]). Severe malaria occurs when the parasite infection overtakes the patient's organs or causes serious problems in the blood stream. Manifestations of severe malaria can include cerebral malaria, severe anemia from hemolysis, pulmonary edema, cardiovascular collapse, and acute kidney failure (CDC [updated 2006]).

Five different species of *Plasmodium* parasites can cause malaria in a human host: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Of these species, *P. vivax*

and *P. falciparum* are the most widespread, and *P. falciparum* is primarily responsible for human deaths (WHO [updated 2009]). Thus, most malaria research is focused on the *P. falciparum* species of malaria parasites. While malaria is preventable and treatable, much of the population in at-risk areas of the world has limited access to healthcare and lack the financial resources needed for anti-malarial drugs. Additionally, parasite resistance to the common anti-malarial drugs chloroquine, sulphadoxine-pyrimethamine, and mefloquine has arisen by spontaneous point mutations and gene duplications, and therefore continued research on new drug candidates, novel drug combinations, and vaccine candidates is crucial (White et al. 2009). Currently there is no vaccine against malaria, though some vaccine candidates are undergoing clinical trials (Goodman and Draper 2010).

The full life cycle of malaria parasites consists of multiple stages in both the mosquito and human hosts (Fig. 1A). *P. falciparum* parasites enter a human host when an infected female *Anopheles* mosquito takes a blood meal and injects sporozoites into the human. The sporozoites travel through the body's bloodstream from the site of injection to the liver. Once in the liver, sporozoites invade hepatocytes and reproduce asexually for a time period of 1 to 2 weeks, producing as many as 30,000 merozoites (Jones and Hoffman 1994). When infected hepatocytes burst, merozoites are released and enter the bloodstream to invade red blood cells. This begins the parasite's intraerythrocytic or asexual life cycle. During the intraerythrocytic phase, the parasite cycles through the stages of ring, trophozoite, and schizont. Within one 48 hour asexual cycle, one invading merozoite matures to a schizont containing 8-20 new merozoites, causing parasite levels in the bloodstream to increase dramatically (Greenwood et al. 2008).

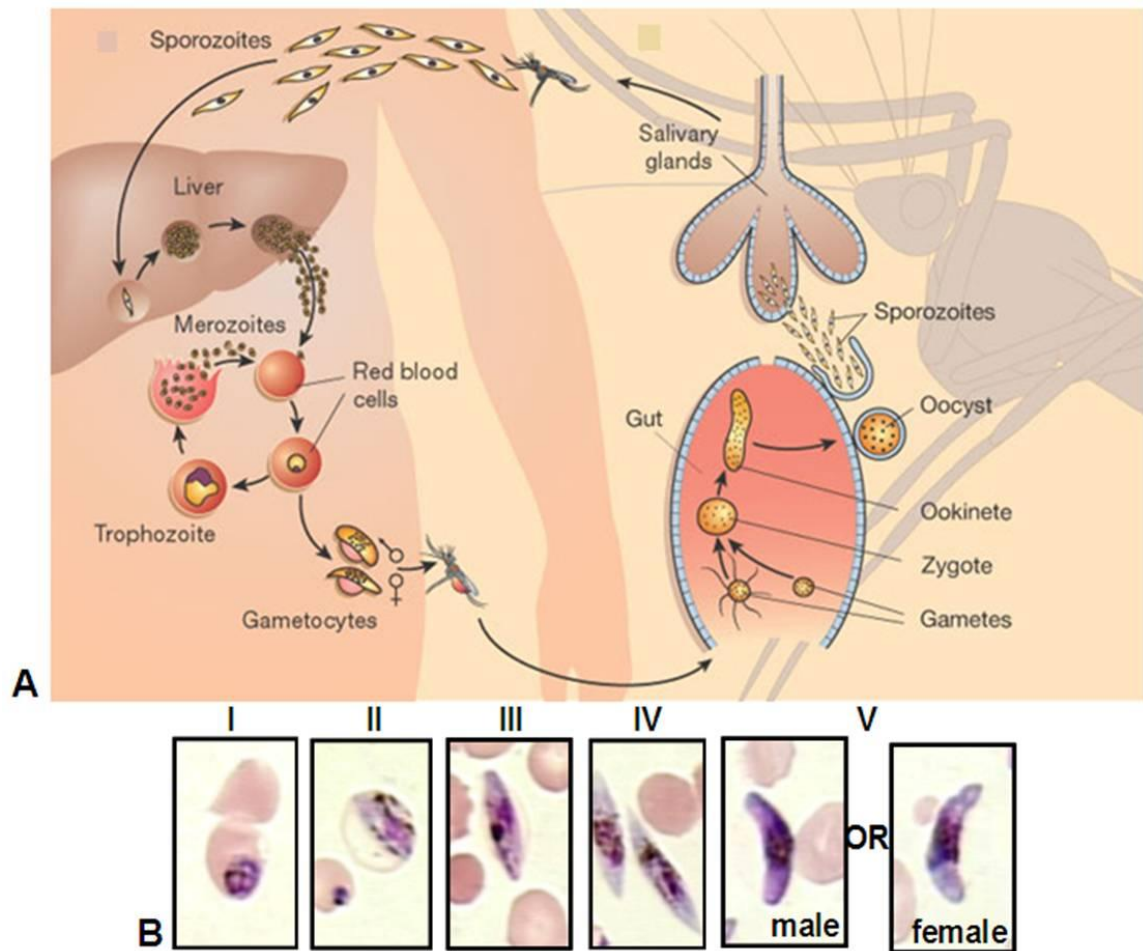


Figure 1. *Plasmodium falciparum* life cycle (A) (Wirth 2002) and five gametocyte developmental stages (B).

At some point during the asexual blood cycle, a small subpopulation of parasites leaves the asexual phase and enters into sexual differentiation, also known as gametocytogenesis. Parasites undergoing sexual differentiation into gametocytes go through five developmental stages (Fig. 1B). Stage I gametocytes are round in shape and indistinguishable from round trophozoites by microscopy. Stage II gametocytes elongate in the host erythrocyte and become “D” shaped. Stage III gametocytes further elongate in the erythrocyte and have curved ends. Stage IV gametocytes appear very elongated and thin with pointed ends, with different pigmentation distribution between male and female parasites. Mature stage V gametocytes are “sausage” shaped with rounded ends; male and female gametocytes are easily distinguishable at this stage by their pigmentation and color, with males appearing pink with scattered pigment and females appearing violet with dense pigment.

These mature gametocytes are then picked up when a mosquito bites the infected individual, and once in the mosquito, the abrupt environmental change triggers the gametocytes to quickly become male and female gametes (Wirth 2002). In the mosquito, the male gametocyte emerges from the red blood cell and transforms into eight motile microgametes, while the female gametocyte emerges from the red blood cell as a round macrogamete (Alano 2007). The male and female gametes fuse in the mosquito midgut to become a zygote, which then develops into an ookinete; the motile ookinete crosses the wall of the gut and transforms into a sporozoite-filled oocyst (Wirth 2002). The oocyst eventually bursts and the released sporozoites make their way to the mosquito’s salivary glands, from where they will be injected into the next human host (Jones and Hoffman

1994). Understanding the parasites' development throughout all of its life cycle stages is necessary to determine methods to stop the transmission of *P. falciparum* malaria.

While much is known about the stages of sexual differentiation, the molecular and genetic basis for the parasites vital transition from the asexual cycle to gametocytogenesis is not understood, though it is assumed that genetic cues exist. It is hypothesized that environmental cues from the human host environment as well as some forms of drug treatment can induce gametocytogenesis (Talman et al. 2004). The exact point of commitment to sexual differentiation is also unclear, though it has been shown that all merozoites emerging from a single schizont will either become gametocytes or will continue on in the asexual cycle (Bruce et al. 1990). Additionally, the gametocytes coming from one schizont are either all male or all female, suggesting that the trophozoite from the previous asexual generation was already committed to sexual development (Talman et al. 2004).

Malaria parasites must enter the sexual cycle to become gametocytes, and only mature gametocytes can survive the mosquito environment and eventually be transmitted to a human host. Blocking this transition or somehow altering the development or function of gametocytes that are produced could prevent the production of mature gametocytes, thus halting malaria transmission. Drug and vaccine candidates that target the gametocyte stages are categorized as transmission-blocking, as the goal of these treatments is to break the crucial link of gametocytes between the human and mosquito hosts (Alano 2007). While transmission-blocking vaccines would not immediately benefit the vaccinated individual, widespread distribution of the vaccine would reduce and eventually eliminate parasite transmission (Greenwood et al. 2008). Some sexual stage



genes and their respective proteins, namely Pfs230 and Pfs48/45, have been identified as potential vaccine targets, as antibodies against these proteins demonstrate some transmission-blocking activity (Williamson 2003). Continued research is necessary to gain a better understanding of the genetic cues necessary for gametocytogenesis and to identify more specific genes as transmission-blocking candidates.

Microarray analysis of the *P. falciparum* transcriptome throughout its life cycle, specifically analyzing gene expression during the stages of gametocytogenesis, is a powerful tool to identify genes that are up-regulated during the sexual cycle and may be crucial in initiating or maintaining normal sexual differentiation. Microarrays utilize an array of thousands of microscopic spots of DNA oligonucleotides on a microarray chip, which are able to hybridize to the sample of target RNA molecules. This hybridization is detected and quantified by fluorescent probes. Microarray data obtained from a Young et al. study in 2005 has provided the mRNA expression profiles of many *P. falciparum* genes. Young et al. collected high purity stage II-V gametocytes and profiled their transcriptomes using a full-genome high-density oligonucleotide microarray (2005). Another study by Silvestrini et al. used microarray analysis to identify genes that are specifically upregulated at the onset of gametocytogenesis (2005). They did this by conducting a comparative microarray analysis with two parasite strains, 3D7 and its gametocyteless derivative clone F12, to identify 117 genes that were differentially expressed in the two clones after sexual induction. Eksi et al. in 2005 also used microarray techniques to identify six members of a previously uncharacterized 36 gene *P. falciparum*-specific subtelomeric superfamily found to be upregulated in early gametocytes. Similarly to the Silvestrini et al. study, this study used microarray analysis

to compare gene expression between two clonal populations derived from 3D7 parasites, one that produced gametocytes and one that produced very few if any gametocytes (Eksi et al. 2005). The Young et al. microarray data is a convenient source of information to quickly construct relative mRNA expression graphs, which can be used to observe the general expression pattern of genes of interest throughout the parasites' life cycle stages in the human host.

A whole-genome microarray was also used to compare the genetic expression differences between a gametocyte-deficient parasite strain (G-) and a parasite strain that produced a high level of gametocytes (G+) (unpublished data Eksi et al.). This microarray data identified a deletion in chromosome 9 in the G- strain; the deleted region contained a single annotated gene, PFI1710w, named *Pfgdv1*, for *P. falciparum* gametocyte development 1 (unpublished data Eksi et al.). Additionally, comparative expression analysis of the G+ and G- strains identified a group of 32 *P. falciparum* genes that are down-regulated in the absence of *Pfgdv1*. These genes have much higher expression levels in the G+ strain and include some previously identified sexual stage-specific genes as well as other uncharacterized genes that are excellent candidates for future study.

One such gene is PFL2550w, which was found to be expressed on average 37.8 times higher in the G+ strain at low parasitemia and 55.5 times higher in the G+ parasite strain at high parasitemia compared to its expression in the G- parasite strain. This significant differential expression along with information attained about PFL2550w on PlasmoDB.org led to the selection of this gene for this research project. From the Young et al. microarray data, an mRNA expression profile graph of PFL2550w was made to

observe its gene expression throughout the parasites' life cycle (Fig. 2). The graph shows varied expression in asexuals, relatively low expression in 3D7 strain parasites, and a slight peak in expression at the beginning of gametocytogenesis in NF54 strain parasites, a gametocyte-rich strain. From data on the PlasmoDB website, PFL2550w is known to contain a DnaJ domain, as well as a secretory signal sequence and an erythrocyte export domain.

DnaJ proteins are members of the HSP40 (Heat Shock Protein – 40 kDa) family of molecular chaperones, a family of proteins that regulates the activity of HSP70 proteins and aids in protein folding (Walsh et al. 2004). DnaJ was first identified in *E. coli* as a molecule that interacted with DnaK (or HSP70) proteins by regulating their ATPase activity (Watanabe 1997). The J domain, a 70 amino acid sequence at the N-terminus, is conserved in all proteins classified as DnaJ proteins and is believed to be the domain that specifically interacts with the HSP70 proteins (Caplan et al. 1993). The J domain consists of four alpha helices, the second of which has a charged surface including at least one pair of basic amino acids, which is essential for interacting with the ATPase domain of HSP70 (Walsh et al. 2004).

Besides their well-known chaperone activity, DnaJ proteins are believed to have other cellular roles. As “heat shock” proteins, they are known to function in the cell's response to stress. Other DnaJ cellular functions identified include mediation of protein degradation, participation in exocytosis and endocytosis, participation in protein translocation, and a potential role in mRNA translation (Cheetham and Caplan 1998). The DnaJ domain is an intriguing characteristic of PFL2550w. In a 2007 study by Botha et al., a large number of HSP40 proteins from *P. falciparum* and other apicomplexa were

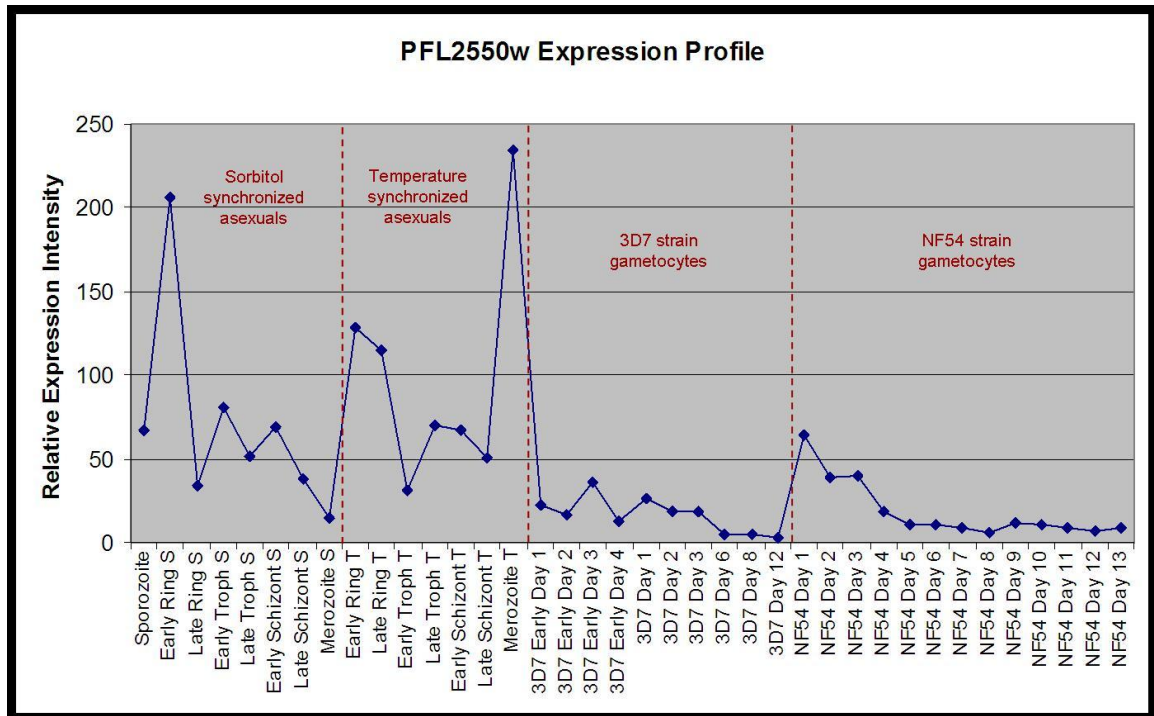


Figure 2. Expression profile of PFL2550w throughout the asexual and sexual *P. falciparum* blood stages.

analyzed. At least 43 *P. falciparum* genes are known to code for members of the HSP40 family, which are further characterized into four types of HSP40 proteins, types I-IV, based on their sequence domain conservation. Only two *P. falciparum* HSP40 type I proteins have been identified; the type I HSP40 proteins show full HSP40 domain conservation with human type I HSP40 protein, having all four domains conserved, and are predicted to function in standard co-chaperone processes. Nine type II HSP40 *P. falciparum* proteins have been identified. The type II HSP40 proteins lack the zinc-binding domain but are functionally similar to type I HSP40s. Twenty type III HSP40 proteins were identified in this study; type III HSP40s do not have domain conservation and only exhibit the signature J-domain. Type III HSP40 proteins are much more divergent than type I and II HSP40s in both cellular localization and function. Type IV HSP40 proteins were previously characterized as part of the type III group but were differentiated into a fourth type in this study (Botha et al. 2007).

PFL2550w was among the proteins studied and was classified as a type IV HSP40 protein. *P. falciparum* encodes twelve type IV HSP40 proteins, which differ from other HSP40 types because of variations in the typically conserved His-Pro-Asp (HPD) motif between helices II and III (Botha et al. 2007). The conserved HPD motif is necessary for the normal interaction between HSP40 and HSP70 proteins, and it is believed that type IV HSP40 proteins have a more complex regulation of HSP70s (Botha et al. 2007). The genome of *P. falciparum* encodes six HSP70 proteins, five of which are homologous to human HSP70 proteins; these HSP70 proteins are believed to interact with many of the type I-III HSP40 proteins in the parasite (Botha et al. 2007). Along with PFL2550w, the *P. falciparum* RESA (Ring-infected Erythrocyte Surface Antigen) proteins and the *P.*

*falciparum* erythrocyte membrane protein (PfEMP2) are classified as type IV HSP40s (Botha et al. 2007). In *P. falciparum*, RESA contains a sequence homologous to the J domain and is known to be released from the dense granules when malaria parasites invade erythrocytes (Watanabe 1997). As a type IV HSP40 protein, we hypothesize that PFL2550w has a complex HSP70 regulatory function and perhaps other chaperone-type functions including protein trafficking and folding.

Heat shock proteins are fascinating to study in *P. falciparum*, as it can be speculated that HSP40 and HSP70 malaria proteins may play important “heat shock” roles in the parasite’s adaptation to its changing thermal environment within its hosts (Watanabe 1997). Another type of heat shock protein from the HSP100 family, HSP101, has been studied in malaria parasites. This protein was found to be part of a novel translocon of exported proteins (PTEX) in *P. falciparum* and is believed to have the specific function of unfolding and feeding denatured proteins through the central channel of the export machine to the red blood cell (de Koning-Ward et al. 2009). As PFL2550w is a hypothetical heat shock protein with a J domain, and also is known to have a secretory signal sequence and an erythrocyte export domain, it is hypothesized that *P. falciparum* PFL2550w protein functions in protein trafficking and regulation within the host erythrocyte.

When the *P. falciparum* parasite invades a red blood cell, it resides within a parasitophorous vacuole and has a significant effect on its host erythrocyte. The host cell becomes rigid and spherical, membrane-bound organelles are formed to enable parasite-host cell interactions, and a variety of *P. falciparum* proteins are exported from the parasitophorous vacuole to the host cell (Bonney and Ménard 2008). Many of the

parasite's exported proteins are expected to be the mediators of these significant changes in the host red blood cell (Sargeant et al. 2006). These alterations to the host cell are necessary for the parasite's virulence and survival. The predicted *P. falciparum* proteome in its most expanded form is predicted to be made up of 420 to 450 proteins, and is approximately 5-10 times larger than the proteomes of other malaria parasites, which implicates *P. falciparum*'s unique pathogenicity and ability to sequester in tissues and organs (Bonney and Ménard 2008). This large group of exported proteins plays various virulence-related roles in the erythrocyte.

One of the most well-studied exported *P. falciparum* proteins is PfEMP1 (*P. falciparum* Erythrocyte Membrane Protein 1), which is encoded by a family of *var* genes. PfEMP1 is trafficked by other exported proteins to the infected red blood cell surface and is anchored there by knobs, complexes made up of the knob associated histidine-rich protein (KAHRP) (Maier et al. 2008). PfEMP1 is believed to have two key roles in parasite infection: antigenic variation and adhesion for the sequestration of infected red blood cells (Craig and Scherf 2001). The antigenic variation of PfEMP1 translation is one of the protein's most intriguing characteristics, as well as one of the malaria parasite's most cunning methods of immune evasion. The *P. falciparum* parasite expresses only one PfEMP1 gene on the infected erythrocyte surface at any one time, but has the ability to switch to a different PfEMP1, expressed by a different *var* gene, to escape the antibody response of the host (Bonney and Ménard 2008). There are also other PfEMP-related proteins, including PfEMP2. PfEMP2, also known as MESA (Mature-parasite-infected Erythrocyte Surface Antigen), is a large protein that accumulates under the plasma membrane underneath knobs of infected erythrocytes (Saul et al. 1992). Many of the

other exported *P. falciparum* proteins are believed to participate in the parasites' virulence by trafficking the necessary virulence proteins, such as PfEMP1, to their correct locations in the RBC cytoplasm and cell membrane (Maier et al. 2008). Erythrocyte membrane proteins PfEMP1 and PfEMP2, along with other exported proteins, are necessary for malaria's virulence and survival in its host environment.

In summary, PFL2550w has been selected for this research project due to its many interesting characteristics. The PFL2550w RNA was found to be expressed at significantly higher levels in G+ parasite cultures compared to G- parasite cultures. Its protein sequence is known to contain a DnaJ domain, an erythrocyte export domain, and a secretory signal sequence. Further, PFL2550w has already been included in a few studies, such as the 2008 study by Maier et al. on exported proteins that are required for virulence, in which PFL2550w was classified as nonessential for asexual growth. While some information is known about this gene and its respective protein, the exact cellular localization and more importantly the function of the protein remains unknown.

In this study of PFL2550w, antibodies will be prepared in mice against the recombinant PFL2550w protein. These antibodies will be used in both western blot and IFA analyses to identify the exact location of the protein in the host erythrocyte and the precise stages of the life cycle when the protein is present. Colocalization IFAs will be done using Pfs16, a known sexual stage protein, and SBP1, a parasite protein associated with Mauer's clefts in the infected red blood cell, to further discover the localization of the PFL2550w protein (Epp and Deitsch 2006). Additionally, an immunoprecipitation will be done to determine what proteins PFL2550w directly interacts with. The phenotype of parasites without the complete PFL2550w gene will also be studied.



Knockout and gene truncation constructs will be prepared for separate transfections into 3D7 strain parasites. Both knockout and truncation constructs were prepared as attaining gene-truncated *P. falciparum* transfectants takes less time and is more often successful than attaining gene knockout transfectants. Gene knockouts, however, provide more concrete results of gene function, and thus were also prepared for this study. The phenotypes of these transfected parasites will give insights into the molecular role of the PFL2550w gene and protein. We hypothesize that PFL2550w will localize to the infected erythrocyte and will have some type of chaperone role in protein trafficking or protein folding.

*P. falciparum* gene PFF0750w was also selected for this research study. It was chosen for further analysis because its mRNA expression pattern was very similar to the expression pattern of PFI1710w, named Pfgdv1, the gene that was found to be deleted in the cultured strain of gametocyte-deficient parasites (unpublished data Eksi et al.). The Young et al. microarray data was again used to prepare an expression profile graph of PFF0750w to observe its expression throughout the parasites' life cycle in the human host (Fig. 3). Another graph was prepared from the same data to show the PFF0750w expression profile in comparison with that of PFI1710w/Pfgdv1 (Fig. 4). The expression profiles of both genes show a peak of increased expression in early schizonts, as well as steadily rising expression throughout the stages of gametocytogenesis. The close similarity of these mRNA expression profiles, suggesting the co-regulation of these genes or possible gene product interaction, was one of the primary factors that have directed further research to the PFF0750w gene. Additionally, PFF0750w is a predicted cyclin-dependent kinase and its amino acid sequence is similar to that of *cdc2* (cell division

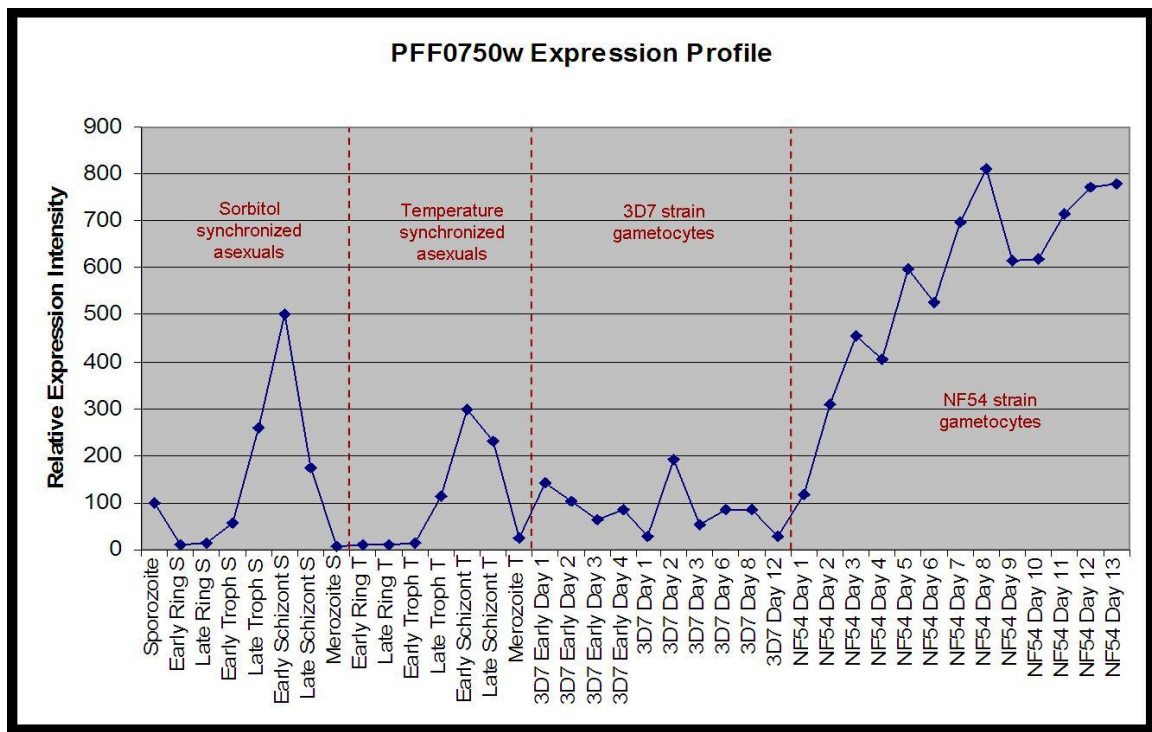


Figure 3. Expression profile of PFF0750w throughout the asexual and sexual *P. falciparum* blood stages.

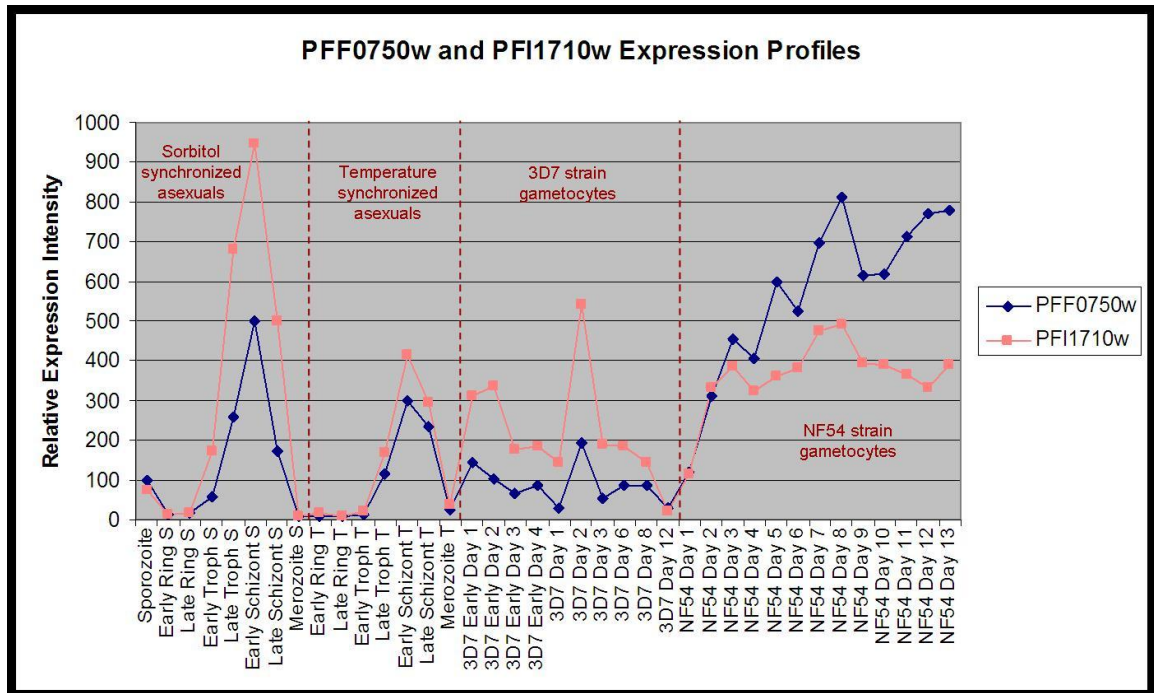


Figure 4. Expression profiles of PFF0750w and PFI1710w throughout the asexual and sexual *P. falciparum* blood stages.

cycle 2) found in yeast (PlasmoDB [updated 2010]). Therefore, PFF0750w may play an important role in regulating the parasites' transcription and cell cycle events.

Research directed towards cell cycle regulation genes and proteins is an important aspect of malaria research, since the molecular and genetic basis for malaria parasites' transition from the asexual cycle to the sexual cycle in the human blood stages is not understood. Interfering with the parasites' cell cycle may prove to be an effective way of controlling malaria transmission, as the transition from the asexual to sexual cycle must involve some assortment of cell cycle control elements. The parasite must tightly regulate its cell cycle and cell division in the host environment to properly complete its life cycle (Merckx et al. 2003).

In a normal eukaryotic cell, there is progression through the G<sub>1</sub> (cell contents duplicated), S (DNA duplicated), G<sub>2</sub>, M (mitosis), and cytokinesis phases of the cell cycle. In *P. falciparum*, there is not a clear correlation between the parasite's life cycle and normal cell cycle events. It is presumed that invading merozoites are in the G<sub>1</sub> phase, where they prepare for duplication, and DNA synthesis (S phase) is initiated in the trophozoite (Merckx et al. 2003). In schizogony, multiple rounds of DNA replication occur asynchronously, forming multinucleate schizonts, without cytokinesis (Striepen et al. 2007). At some point, a subset of parasites leaves the "cell cycle," potentially during the G<sub>1</sub> phase, and remain in G<sub>0</sub> as single organisms that will go on to gametocytogenesis. It remains to be determined how the parasite's gene expression and other cell cycle regulators such as protein kinases participate in directing the parasite's unique cell cycle events.

Protein kinases are known to play a vital role in cell cycle control, a fact that has been well studied in yeast, and are also suggested to be significant in malaria cell cycle control (Pelech et al. 1990). The role of protein kinases as cell cycle control molecules is to regulate many biochemical processes; they are kinase enzymes that modify other proteins by chemically adding phosphate groups to them. In the past few decades of malaria research, several malaria protein kinases belonging to the Ser and Thr superfamily of kinases have been isolated and studied (Kappes et al. 1999). Functional predictions for these proteins have been speculated based on their kinase group classification and genetic similarity to kinases in other organisms, but these predictions may not be accurate due to *Plasmodium*'s evolutionary divergence from yeast and the other higher eukaryotes to which its genes are being compared (Kappes et al. 1999).

In fission yeast, the transition into the S phase and mitosis is regulated by the p34<sup>cdc2</sup> serine/threonine protein kinase, which is encoded by the *cdc2* gene (Doerig et al. 1995). In higher eukaryotes, p34<sup>cdc2</sup> regulates the entry into mitosis, while the transition into the S phase is regulated by other cyclin-dependent kinases (CDKs) (Doerig et al. 1995). CDKs have been identified in all eukaryotes studied thus far and are usually involved in cell cycle regulation (Kappes et al. 1999). CDKs typically regulate transcription and also play a role in RNA processing. In higher eukaryotes, to be activated and perform its regulatory function in the cell, the CDK subunit requires the binding of a regulatory partner, a cyclin (Kinnaird and Mottram 1997). Many CDK-like and cyclin-related kinase genes have been identified and studied in *P. falciparum*, including PfPK5, Pfmrk, CDK/MAPK hybrid protein PfPK6, Pfcrk-1, Pfcrk-3, Pfcrk-4, and Pfcrk-5 (Geyer et al. 2005). Many of these genes have high sequence homology to

known kinases in other organisms (Geyer et al. 2005). Pfpk5 and Pfmrk have been extensively studied and most likely have crucial functions in cell cycle control and differentiation, making them attractive anti-malarial drug candidates (Geyer et al 2009). As some CDK inhibitory drugs have been found to impede parasite growth, further research is needed to understand the specific cellular function of all *P. falciparum* CDKs.

PFF0750w codes for a protein that is classified as a predicted cyclin-dependent protein kinase (PlasmoDB [updated 2010]). The PFF0750w gene was previously identified on PlasmoDB.org as a *cdc2*-like kinase due to its sequence homology to the *cdc2* gene in *S. pombe*. PFF0750w, formerly named Pfcrk-5 and MAL6P1.271, was also identified in a 2004 study by Ward et al. as part of the malarial CMGC group of kinases (Cyclin-dependent, Mitogen-activated, Glycogen synthase, and CDK-like kinases), specifically one of six in the cyclin-dependent kinase family. The phylogenetic trees created for this study show that PFF0750w is related to both CDKs and MAPKs, mitogen-activated protein kinases (Ward et al. 2004). As the exact function of this kinase in the parasite cell cycle is not known, further study is necessary.

Since PFF0750w may be influential in cell cycle regulation as a cyclin-dependent kinase, it may be a significant gene in the parasites' life cycle. The transcription profile generated from the Young et al. data shows an increase in mRNA expression levels throughout gametocytogenesis in NF54 strain parasites (2005). Additionally, PFF0750w is known to have a similar expression pattern to Pfgdv1, the gene deleted from a gametocyte-deficient strain of parasites, as was mentioned previously. Since the two genes are expressed so similarly throughout the *P. falciparum* life cycle, there may be some interaction between the gene products, which could suggest that the removal of

PFF0750w may also result in deficient gametocyte production. The specific cellular function and localization of PFF0750w remains unknown; in this study, we will attempt to determine the cellular role of the PFF0750w gene and the localization and function of the PFF0750w protein. 3D7 parasites will be transfected with a PFF0750w gene truncation construct and then cloned to isolate a population of parasites that have correctly integrated the gene truncation plasmid. The PFF0750w gene truncation parasites will be analyzed to see in what ways their life cycle is affected by the loss of a functional PFF0750w protein. We hypothesize that the absence of the PFF0750w protein from the parasite will have a harmful effect on the parasite's progression through its life cycle. Depending on which life cycle stages are interrupted, it may be difficult or impossible to grow transfectant parasites in culture. Separately, the coding region of PFF0750w will also be prepared in an expression plasmid and used to generate recombinant protein for antibody production in mice. These antibodies will be used in both western blots and IFAs to determine the exact location of the protein and the precise stages of the life cycle when the protein is present.

## **CHAPTER II**

### **MATERIALS AND METHODS**

#### Experimental Design

A similar experimental design was used for gene and protein studies on PFL2550w and PFF0750w. To study the functions of these genes, gene truncation plasmid constructs were prepared, containing a segment of the 5' flanking region and the 5' end of the gene, as well as a drug-selectable marker region. Additionally for the PFL2550w gene, a gene knockout construct was prepared in a plasmid containing two drug-selectable markers. These constructs were transfected into 3D7 strain parasites in separate transfections. The parasites were cultured in media containing the drug corresponding to the selectable marker(s) of the plasmid to eliminate parasites that did not integrate the plasmid. After a period of time following the transfections, a mixed culture of episomal, wild type, and correctly integrated parasites grew up. From these mixed cultures, limited dilution cloning was done to isolate a clonal population of parasites that had correctly integrated the gene truncation or knockout plasmid.

To study the function and localization of the PFL2550w and PFF0750w proteins, constructs were separately prepared containing the coding regions of both genes for production of maltose binding protein (MBP) fusion recombinant proteins. *E. coli* were transformed with these plasmids and protein expression was induced. The recombinant proteins were purified by affinity chromatography and used for mice injections. After



three injections, tail bleeds were performed on the mice to collect the antiserum. The antiserum was analyzed by western blots, immunofluorescence assays (IFA), and immunoprecipitation assay.

### DNA Amplification

To prepare the gene truncation and gene knockout constructs, polymerase chain reaction (PCR) was done using a PTC-100 Programmable Thermal Controller [MJ Research Inc.; Watertown, MA] to amplify the target DNA regions for insertion into the respective bacterial plasmid. The PFL2550w gene is found on *P. falciparum* chromosome 12, from base pair (bp) position 2,168,166 to 2,169,744. For the PFL2550w gene truncation construct, PCR was used to amplify a 615 bp region inside the PFL2550w gene on chromosome 12 of the 3D7 strain *P. falciparum* genomic DNA, between bp position 2,168,384 and 2,168,996. The primers included *Sac*II and *Spe*I restriction enzyme sites at the 5' and 3' ends, respectively.

For the PFL2550w gene knockout construct, two separate fragments of DNA, the F1 fragment on the 5' end of the gene and the F2 fragment on the 3' end of the gene, were amplified separately. For the F1 fragment, PCR was used to amplify a 906 bp region, which included the 5' untranslated region of the gene as well as the 5' end of the PFL2550w gene, on chromosome 12 of 3D7 strain *P. falciparum* genomic DNA, between bp positions 2,167,326 and 2,168,229. The primers included *Sac*II and *Spe*I restriction enzyme sites at the 5' and 3' ends, respectively. For the F2 fragment, PCR was used to amplify a 741 bp region on the 3' end of the PFL2550w gene on chromosome 12 of 3D7 strain *P. falciparum* genomic DNA, between bp 2,168,981 and 2,169,720. The primers included *Nco*I and *Avr*II restriction enzyme sites at the 5' and 3' ends, respectively.

The PFF0750w gene is found on *P. falciparum* chromosome 6, from bp 644,048 to 646,165. To prepare the PFF0750w gene truncation construct, PCR was used to amplify a 824 bp region at the 5' end of PFF0750w on chromosome 6 of *P. falciparum* genomic DNA, between bp 644,000 and 644,824. The primers included *Sac*II and *Xba*I restriction enzyme sites at the 5' and 3' ends, respectively. The primers for this reaction were prepared by students from Dr. Jutta Heller's BIOL 390 class at Loyola University Chicago in the fall semester of 2007.

Each PCR amplification reaction was done with four replicates, and each reaction was carried out in a 0.2 ml PCR tube, using the following amounts of reagents per tube:

- 0.5 µl dNTPs (1.25 mM)
- 1.5 µl forward primer (600 ng/µl)
- 1.5 µl reverse primer (600 ng/µl)
- 3 µl 3D7 strain *P. falciparum* genomic DNA (1 µg/µl)
- 5 µl 5X Green GoTaq® Buffer
- 0.5 µl GoTaq® DNA Polymerase (5 units/µl)
- 13 µl dH<sub>2</sub>O

The tubes were then placed into the thermocycler and the typical reaction was:

- 1) Denaturation: 94°C for 30 seconds
- 2) Annealing: 50°C for 30 seconds, 46°C for 30 seconds
- 3) Extension: 62°C for 1 minute
- 4) Repeat steps 1-3 35 times
- 5) Extension: 62°C for 5 minutes
- 6) Preservation: Remain at 10°C

After each PCR experiment, 5 µl PCR product from each tube was run with buffer and dH<sub>2</sub>O on a 0.8% agarose/TAE gel to verify PCR results. The positive PCR products from replicate tubes were combined and purified using the QIAquick® PCR Purification Kit, following the kit's protocol for PCR purification [Qiagen; Valencia, CA].

## Bacterial Vectors

The bacterial vector used for the PFL2550w and PFF0750w gene truncation constructs was pDT.Tg23 (Wu et al. 1996). This vector is 7.1 kb in length and contains a *dhfr-ts* coding region from the *T. gondii* M2M3 mutant gene that confers resistance to the drug pyrimethamine. The vector has a polylinker site that was digested with restriction enzymes *Sac*II and *Spe*I for the PFL2550w construct and separately digested with *Sac*II and *Xba*I for the PFF0750w construct.

The bacterial vector used for the PFL2550w gene knockout construct was pHHT-FCU (Maier et al. 2006). This vector is 7.6 kb in length and contains two selectable cassettes. A human *dihydrofolate reductase* (*hDHFR*) region is included as a positive selectable marker driven by the *calmodulin* promoter, which confers resistance to the drug WR99210. The second cassette consists of the *S. cerevisiae* cytosine deaminase/uracil phosphoribosyltransferase (*ScFCU*) chimeric gene for negative selection with 5-fluorocytosine (5-FC). The expression of the negative selection cassette is driven by the heat shock protein 86 promoter, and it is flanked by the *P. berghei dhfr* terminator. This plasmid was first digested with *Sac*II and *Spe*I to prepare for the insertion of the F1 fragment of PFL2550w into the plasmid. After this fragment was confirmed to be properly inserted, the vector was digested with *Nco*I and *Avr*II for the insertion of the F2 fragment of PFL2550w.

## Digestion

The purified PCR products and the bacterial vectors were digested so that the

recombinant plasmids could be ligated together. The digestions were typically carried out as follows:

PCR product digest

5 µl PCR product  
2 µl 10X enzyme specific buffer  
2 µl Bovine Serum Albumin (BSA) (0.1 mg/ml)  
0.5 µl restriction enzyme 1 (20,000 units/ml)  
0.5 µl restriction enzyme 2 (20,000 u/ml)  
10 µl dH<sub>2</sub>O

2 hours at 37°C.

Vector digest

3 µl plasmid  
2 µl 10X enzyme specific buffer  
2 µl BSA (0.1 mg/ml)  
0.5 µl restriction enzyme 1 (20,000 u/ml)  
0.5 µl restriction enzyme 2 (20,000 u/ml)  
12 µl dH<sub>2</sub>O

2 hours at 37°C.

The corresponding restriction enzymes for each PCR product and each vector were used as restriction enzymes 1 and 2, as were mentioned in the DNA Amplification and Bacterial Vectors sections previously. The vectors were further digested with 0.2 µl bacterial alkaline phosphatase (30 units/ µl) for 30 minutes at 65°C to prevent the cut vector ends from re-ligating.

Agarose Gels

After digestion, the digested PCR products and plasmids were run on 0.8% agarose gels with 0.028% guanidine to size fractionate the DNA. The gels were made in TAE (40 mM Tris-acetate; 1 mM EDTA) buffer, with 0.001% ethidium bromide (EtBr) added to the gel to allow the DNA to be visualized with ultraviolet light. The 0.028% guanidine was included in the gel to prevent fragmentation of the DNA when it was

viewed with the ultraviolet light. Each digestion (20 µl) was run in a separate lane with 4 µl DNA loading buffer (15% glycerol, 0.125% bromophenol blue). Size standards were run in a separate lane to verify the size of the DNA pieces. The gels were typically run for 60 minutes at 120 volts.

### Ligation

After electrophoresis, the agarose gels were viewed with ultraviolet light and the bands corresponding to the digested products and vectors were excised from the gel using a razor blade. These gel pieces were placed in separate Eppendorf tubes and could be stored at -20°C for a short period of time. The digested DNA was extracted from the gel pieces using the MinElute™ Gel Extraction Kit, per the kit protocol [Qiagen]. The extracted DNA was eluted in 10 µl dH<sub>2</sub>O at the end of the protocol and was stored at -20°C.

Each PCR product of amplified DNA from *P. falciparum* was mixed with its corresponding plasmid and ligated together as follows:

- 1 µl purified vector DNA
- 6 µl purified PCR product insert DNA
- 1 µl T4 DNA ligase (400,000 units/ml)
- 1 µl 10X T4 DNA ligase buffer
- 1 µl dH<sub>2</sub>O

Incubate 4 hours or overnight at room temperature.

### Transformation of *E. coli*

For the preparation of the PFL2550w and PFF0750w gene truncation constructs, the following method was used for the transformation of the plasmid into *E. coli*. One µl of a 1:5 dilution of the ligation mixture was added to 20 µl of ElectroMax DH10B™ electrocompetent *E. coli* cells [Invitrogen; Carlsbad, CA] in a sterile Eppendorf tube.

After a 20 minute incubation on ice, 20  $\mu$ l of this mixture was placed in a cold 1 mm gap disposable E.coli Pulser® cuvette [Bio-Rad; Hercules, CA]. The cuvette was placed in a TransPorator™ Plus [BTX; San Diego, CA], and a 1.8 kV electric pulse was used to permeabilize the *E. coli* cells, allowing the entry of the gene truncation construct vectors. Following the electroporation, the cells were grown in 900  $\mu$ l of SOC medium [Invitrogen; Carlsbad, CA] for 1 hour at 37°C, and then 300  $\mu$ l of this mixture was spread on Luria-Broth/Agar plates containing 100  $\mu$ g/ml ampicillin.

The plates were prepared by adding 2.5 g Bacto-yeast, 5.0 g Bacto-tryptone, 5.0 g NaCl, and 7.5 g Bacto-agar to 500 ml ddH<sub>2</sub>O. This mixture was autoclaved and after cooling to 50°C, 50 mg ampicillin was added, and the mixture was poured into tissue culture plates. The plates were stored at 4°C until use and then warmed to 37°C before being used in the experiment.

For the preparation of the PFL2550w gene knockout construct, transformation by electroporation was not effective, so chemically-competent cells were transformed by heat shock. For this transformation, a polypropylene round-bottomed tube was chilled on ice and NZY<sup>+</sup> broth was preheated to 42°C. The NZY<sup>+</sup> broth was previously prepared by combining 5 g NZ amine, 2.5 g yeast extract, and 2.5 g NaCl to enough dH<sub>2</sub>O to achieve a final volume of 1 liter. The pH of the solution was adjusted to 7.5 using NaOH, and the solution was then autoclaved. The broth was allowed to cool and sterile 6.25 ml 1 M MgCl<sub>2</sub>, 6.25 ml 1 M MgSO<sub>4</sub>, and 10 ml 20% (w/v) glucose were added prior to use. While the NZY<sup>+</sup> broth preheated, XL10-Gold® ultracompetent *E. coli* cells [Stratagene, Agilent Technologies; Santa Clara, CA] were thawed on ice. When thawed, 100  $\mu$ l cells were added to the chilled polypropylene tube. 4  $\mu$ l of XL10-Gold®  $\beta$ -Mercaptoethanol

mix was added to the aliquot of cells, and the tubes were swirled gently and incubated on ice for 10 minutes. 2 µl of the PFL2550w gene knockout ligation mixture was added to the cells. The tubes were swirled gently and incubated on ice for 30 minutes. The tubes were then heat pulsed in a 42°C water bath for exactly 30 seconds, followed by incubation on ice for 2 minutes. 900 µl of the preheated NZY<sup>+</sup> broth was added to the cells, and the tube was then incubated for 1 hour at 37°C. After the hour incubation, 200 µl of the mixture was spread on Luria-Broth/Agar plates containing 100 µg/ml ampicillin.

For both transformation methods, the LB/amp plates spread with the transformation mixture were placed in a 37°C incubator overnight. Only *E. coli* cells containing the gene alteration vectors should be able to grow in the presence of ampicillin. The following day, single colonies were picked from the plates and grown up in polypropylene tubes containing 3 ml Luria-Broth media and 0.3 mg ampicillin for 24 hours at 37°C.

### Colony PCR

Due to the high number of *E. coli* colonies found to be negative in the preparation of the PFL2550w gene knockout construct, a method known as Colony PCR was used to screen a large number of colonies at a time to find those containing the gene knockout construct. Colony PCR uses primers specific to the construct of interest to amplify a region of the construct, which will then show which colonies contain the construct. Patch plates were prepared by spreading small patch regions of 30-35 colonies on a new LB/amp plate, with each patch correlated to a number written on the outside of the plate. The patch plates were grown overnight at 37°C. From the patch plates, colonies were

picked using separate pipette tips, and three colonies at a time were suspended in 20  $\mu$ l dH<sub>2</sub>O in an Eppendorf tube. All the Eppendorf tubes were boiled at 95°C for 3 minutes to break open the *E. coli* cells. 2x PCR mix was prepared using the following reagents:

- 4  $\mu$ l dNTPs (1.25 mM)
- 10  $\mu$ l forward primer (600 ng/ $\mu$ l)
- 10  $\mu$ l reverse primer (600 ng/ $\mu$ l)
- 20  $\mu$ l 5X Green GoTaq® Buffer
- 2  $\mu$ l GoTaq® DNA Polymerase (5 units/ $\mu$ l)
- 34  $\mu$ l dH<sub>2</sub>O

10  $\mu$ l of the 2x PCR and 10  $\mu$ l of each bacterial suspension were mixed in separate PCR tubes. The tubes were then placed into the thermocycler and were typically run using the same PCR program as was detailed previously. All PCR mixtures were run in separate lanes on 0.8% agarose gels to visualize which colonies had a DNA band present at the approximate size of the amplified region and therefore contained the gene knockout construct. As colonies were combined three to a tube, the three colonies from which a positive band was found in the agarose gel were then grown up in separate polypropylene tubes containing 3 ml Luria-Broth media and 0.3  $\mu$ g ampicillin for 24 hours at 37°C.

#### Plasmid Isolation

The QIAprep® Spin Miniprep Kit [Qiagen] was used to isolate the plasmid constructs from the prepared 3 ml *E. coli* broths, according to the kit protocol. Briefly, the *E. coli* were lysed, genomic DNA was precipitated, and the remaining soluble fraction was applied to a mini-column provided with the kit. Plasmid DNA bound to the column, was washed twice, and then was eluted in 25-50  $\mu$ l dH<sub>2</sub>O.

#### Restriction Analysis

The DNA collected from the Miniprep procedures was digested with the



restriction enzymes that had been incorporated into the constructs, as were mentioned previously, to confirm that the construct DNA had been correctly isolated from the *E. coli* cells and to confirm the presence of the inserted *P. falciparum* DNA in the plasmid constructs. For instance, the PFL2550w gene truncation construct was digested with *SacII* and *SpeI* at 37°C for 1 hour. The digests were run on 0.8% agarose/TAE gels for 40 minutes at 130 V and compared to DNA molecular size standards also run on the gel to determine if the insert DNA was the correct size.

### DNA Sequencing

After the presence of the correct DNA insert in the plasmid was confirmed by restriction analysis, DNA obtained from the Miniprep procedure was sent at a concentration of 0.3 µg/ml to the DNA sequencing facility at the University of Chicago [University of Chicago Cancer Research Center: DNA Sequencing Facility; Chicago, IL]. The sequence reports obtained were evaluated manually and using BLAST software (<http://blast.ncbi.nlm.nih.gov/Blast.cgi> [updated 2010]) to confirm the correct sequences of the inserts and the correct integration of the *P. falciparum* DNA into the plasmids.

### Plasmid DNA Amplification

Once the sequences were confirmed to be correct, the construct DNA was amplified using the QIAGEN® Plasmid Maxi Kit [Qiagen]. 500 ml LB broth and 50 mg ampicillin inoculated with the *E. coli* colony containing the correctly sequenced plasmid was incubated overnight at 37°C for each of the plasmids. The construct DNA was then isolated per the kit protocol and collected in dH<sub>2</sub>O for parasite transfections.

### Parasite Transfection

Electroporation was used to transform *P. falciparum* cells with the PFL2550w and

PFF0750w plasmid construct DNA. Each construct was separately transfected into three parasite flasks each using the following protocol, done in triplicate for each construct. Two days before the transfection, 150 µg plasmid DNA collected from the Maxi prep procedure was precipitated in an Eppendorf tube with 1/10 volumes 3M NaAc (pH 5.2) and 2.5 volumes sterile 100% ethanol overnight at -20°C. One day before the transfection, the precipitated DNA was removed from -20°C and spun at 13,000 rpm for 15 minutes to pellet the DNA. The pellet was washed three times with 70% ethanol and after the last wash, the tube was transferred to the biosafety hood where the ethanol was carefully removed and the pellet was allowed to air dry. Once dry, the DNA was resuspended in 15 µl pre-warmed TE buffer (pH 8.0) and stored at 4°C overnight to ensure that all the DNA dissolved. On the day of the transfection, the DNA in TE was incubated at 37°C for 5 minutes. Cytomix was previously prepared by the following protocol:

120 mM KCl  
0.15 mM CaCl<sub>2</sub>  
25 mM HEPES (pH 7.6)  
2 mM EGTA (ethylene glycol tetraacetic acid) (pH 7.6)  
5 mM MgCl<sub>2</sub>  
10 mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub> (pH 7.6) – added last  
in dH<sub>2</sub>O

385 µl pre-warmed Cytomix was added to the DNA and mixed gently.

On the day of the transfection, a flask of 3D7 strain parasites was smeared to confirm that the parasitemia level was approximately 4-5%. The 3D7 parasites were spun down, and the supernatant was discarded. From the pellet, 200 µl parasitized red blood cells (RBCs) were transferred to a sterile Eppendorf tube. A 25 cm<sup>2</sup> flask was prepared for each transfection containing 10 ml RPMI 1640 w/L-Glutamine + 25 mM HEPES + 50

mg/L Hypoxanthine media [K-D Medical, Inc.; Columbia, MD] and 3% hematocrit. 400  $\mu$ l of the DNA in Cytomix was added to the 200  $\mu$ l parasitized RBCs, and the mixture was transferred to a 0.2 cm electroporation cuvette [Fisher Scientific; Pittsburgh, PA]. The mixture was electroporated in a Gene Pulser® Electroporation System [Bio-Rad] at 0.31 kV, 950  $\mu$ F capacitance, and no resistance for approximately 8 msec. The electroporated mixture was immediately resuspended in 1 ml media and transferred to the prepared flask. The flasks were gassed with a gas mixture of 5.6% CO<sub>2</sub>, 5.0% O<sub>2</sub>, and 89.4% N<sub>2</sub> and were placed in a 37°C incubator.

On the day after transfection, the RPMI media was removed and replaced with 9.5 ml fresh media. On the second day after transfection, the media was again replaced and drug selection was started using the drug that corresponded to each construct. 15 ng/ml pyrimethamine was added at this point to the PFL2550w and PFF0750w gene truncation constructs, as the pDT.Tg23 vector used for those constructs contained a *dhfr-ts* region that confers resistance to pyrimethamine. For the PFL2550w gene knockout construct, in vector pHHT-FCU, 5  $\mu$ M WR99210 drug was added to positively select for parasites containing the plasmid, which contained a *hDHFR* region to confer resistance to this drug. Transfected parasite cultures were maintained by replacing the media and drug daily for the first two weeks, and replacing media and drug every other day after the first two weeks. Additionally, every 7 days 100  $\mu$ l fresh 50% hematocrit RBCs were added. The cultures were maintained until a resistant parasite population had grown up. Once the transfected parasites reached a parasitemia of approximately 5% rings they were frozen, and genomic DNA was prepared for analysis to determine if the plasmid had been correctly integrated into the parasite genome. In the case of the PFL2550w gene knockout

construct in vector pHHT-FCU, after drug resistant parasite cultures had grown up, the cultures were synchronized with sorbitol and then subjected to negative selection with the drug 5-fluorocytosine.

### Integration PCR

To test for proper integration of the PFF0750w gene truncation plasmid into the transfected parasites, genomic DNA from the parasites was extracted using the Promega kit for Genomic DNA Isolation, following the protocol for isolation of genomic DNA from whole blood [Promega; Madison, WI]. DNA was extracted from two independent lines of parasites transfected with the PFF0750w gene truncation plasmid, which were designated J1 and J2. Four primers were prepared and used in four different two-primer combinations to amplify episomal DNA, wild type (WT) 3D7 *P. falciparum* DNA, WT-plasmid integrated at the 3' end, and WT-plasmid integrated at the 5' end. Separate PCR tubes were prepared for each primer pair with each type of DNA for the PCR experiment: J1, J2, WT 3D7 *P. falciparum* as a positive control, and no DNA as a negative control. Tubes were prepared and run in a PCR program as was described previously. The PCR reactions were then run on 0.8% agarose gels for 60 minutes at 120 V.

### Limited Dilution Cloning

After chromosomal integration was confirmed in the J1 and J2 transfectants, limited dilution cloning plates were set up to isolate a clonal, integrated parasite line. To set up a cloning plate, the number of parasites per  $\mu\text{l}$  in the stock culture was determined using the parasitemia of the transfectant culture and the number of RBC/ $\mu\text{l}$  in the stock transfectant culture. The culture was diluted to achieve a concentration of approximately 1 parasite/ $\mu\text{l}$ . Using a 96-well plate, concentrations of 0.5, 1.0 and 2.0 parasites/ $\mu\text{l}$  were

set up, with each concentration in two rows of 10 wells of diluted culture, media, and RBCs. The clonal plates were feed every 2 days and given new RBCs every week. Random wells were smeared every week to check for clonal parasites.

### Protein Construct Preparation

To study the function and localization of the PFL2550w and PFF0750w proteins, constructs were separately prepared containing the coding regions of the genes of interest to be used to produce MBP fusion recombinant protein. For the PFL2550w recombinant protein construct, PCR was used to amplify a 1347 bp region inside the PFL2550w gene of 3D7 strain *P. falciparum* genomic DNA, on chromosome 12 between bp positions 2,168,399 and 2,169,744, using primers incorporating *Xba*I and *Pst*I restriction enzyme sites at the 5' and 3' ends, respectively.

To prepare the PFF0750w recombinant protein construct, PCR was used to amplify a 972 bp region at the 3' end of the PFF0750w gene, on chromosome 6 between bp positions 645,193 and 646,165. This reaction used primers that incorporated *Bam*HI and *Pst*I restriction enzyme sites at the 5' and 3' ends, respectively. The primers for this reaction were prepared by students from Dr. Jutta Heller's BIOL 390 class at Loyola University Chicago in the fall semester of 2007.

The bacterial vector used for both recombinant protein constructs was pIH902 (Williamson et al. 1995). This vector was cut with *Xba*I and *Pst*I for ligation to the PFL2550w PCR product and cut with *Bam*HI and *Pst*I for the PFF0750w protein construct. An identical method was used to prepare the PFF2550w and PFF0750w MBP recombinant protein constructs as was described in the DNA Amplification, Bacterial

Vector, Digest, Agarose Gels, Ligation, Transformation, Plasmid Isolation, Restriction Analysis, and DNA Sequencing sections previously.

### Protein Expression

To express the recombinant protein constructs, a frozen glycerol stock of the vector containing the correct coding region insert was used to inoculate 25 ml of sterile LB medium containing 2.5 mg ampicillin. This bacterial culture was grown in an incubator shaker at 37°C overnight. The next morning, 20 ml of the overnight bacterial culture was added to an autoclaved 2 L Erlenmeyer flask containing 500 ml LB and 250 mg ampicillin. This flask was placed in the incubator shaker and allowed to grow at 37°C until the Optical Density (OD) reading at 600 nm was determined to be between 0.4 and 0.8 using the Spectronic 20 [Bausch and Lomb; Rochester, NY]. 100 µl 1 M Isopropylthio-B-D-galactosidase (IPTG) was added to the culture to remove the *lac* repressor and allow for the induction of the promoter to begin transcription of the gene of interest. The flask was returned to the shaking incubator for 4 hours at 24°C.

After shaking for 4 hours, the contents of the flask were poured into two 500 ml centrifuge tubes, and the tubes were spun at 5000xg for 20 minutes in a Sorvall RC6 Plus Superspeed centrifuge [Thermo Fisher Scientific; Waltham, MA]. The supernatant was removed, and the two pellets were resuspended in three 10 ml aliquots of 1X phosphate buffered saline (PBS) and transferred into the same tube. The centrifuge tube was spun again for 20 minutes at 5000xg. The supernatant was removed, and the pellet was frozen at -20°C overnight.

### Protein Purification

The next day, the frozen pellet was resuspended in 9 ml Lysis buffer (50 mM

Tris-Cl at pH 8.0, 1 mM EDTA, 100 mM NaCl), 0.266 mg/ml lysozyme, and 17.4 µg/ml phenylmethyl sulfonyl fluoride (PMSF). This was incubated on ice for 20 minutes, and then an additional 10 ml of Lysis buffer was added along with 20 µl each of three protease inhibitors, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 2 µg/ml aprotinin. The solution was poured into a 50 ml glass beaker and placed on ice, and the solution was sonicated at least 8 times with a 10 second pulse followed by a 30 second pause, using a Misonix Sonicator® 3000 [QSonica, LLC.; Newtown, CT]. Once the sonicated solution appeared transparent, it was transferred to a 50 ml centrifuge tube, and 108 µl 1 M MgCl<sub>2</sub>, 1.44 g NaCl, and 0.36 mg DNaseI was added. This solution was incubated with rocking for 1 hour at 4°C, and then 10 ml TE Buffer (20 mM Tris at pH 8.0, 1 mM EDTA at pH 8.0) and 10 µl each of 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 2 µg/ml aprotinin were added. The tube was spun at 5000xg for 60 minutes in the Sorvall RC6 Plus Superspeed centrifuge.

During the spin, 2 ml of amylose resin slurry [New England BioLabs; Ipswich, MA] was added to a 20 ml polyprep chromatography column [Bio-Rad] and allowed to drain through. The resin was washed with 5 ml dH<sub>2</sub>O and then with 10 ml Column buffer (20 mM Tris at pH 8.0, 200 mM NaCl, 1 mM EDTA). The bottom of the column was capped, and the resin was resuspended in 2.5 ml TE buffer and transferred to a 50 ml conical tube. The supernatant from the spin of the sonicated solution was added to the washed resin, and the volume was increased to 50 ml with TE buffer and 20 µl each of 1 µg/ml leupeptin, 1 µg/ml pepstatin, 2 µg/ml aprotinin, and 17.4 mg/ml PMSF. The tube was incubated on a rocker at 4°C overnight.

The next day, the entire solution of resin and supernatant was poured directly into the 20 ml column used the day before, and the flow through was collected. The column was then washed three times with 10 ml Column buffer, and each 10 ml aliquot was collected for future analysis. The recombinant protein was then eluted with Column buffer containing 10 mM maltose in ten 1 ml aliquots. These aliquots were stored at -20°C. Eluted aliquots were analyzed by size fractionation on a polyacrylamide gel to confirm the presence and size of recombinant protein using SeeBlue Plus2® Prestained Standard [Invitrogen; Carlsbad, Ca].

#### Estimation of Protein Concentration

The recombinant protein aliquots were quantified using Bio-Rad Protein Assay solution [Bio-Rad]. 10 µl of each eluted protein aliquot was added to a borosilicate glass tube containing 2.5 ml Bio-Rad Protein Assay solution (diluted 1:4), and OD at 595 nm was measured on the Spectronic 20 machine. The protein concentrations were calculated by comparing the protein aliquot OD readings to those obtained from reading standards set up using Bovine Serum Albumin (BSA) at known concentrations in Bio-Rad solution.

#### Mice Immunizations and Tail Bleeds

Inbred CAF1/J standard F1 hybrid mice (The Jackson Laboratory, Animal Resources; Bar Harbor, ME), after being ear-punched for future reference, were vaccinated by inter-peritoneal injection with 50 µg recombinant protein emulsified in Ribi adjuvant, Sigma Adjuvant System™ [Sigma Aldrich; St. Louis, MO]. The adjuvant was prepared by adding 1 ml warm, sterile 1X PBS to each warmed vial of adjuvant. The vial was vortexed [Vortex Genie 2™, Fisher Scientific] for 2 minutes to suspend the entire contents of the vial in PBS. A solution was prepared such that each mouse would



receive 100 µl Ribi adjuvant, 50 µg recombinant protein, and the remaining volume of sterile, warm 1X PBS. Each mouse was injected with 200 µl solution. In total, every mouse was vaccinated three times, with one month between each injection.

One week after the third injection, tail bleeds were performed on the mice and approximately 200 µl blood was collected from each mouse. The blood was centrifuged at 4°C for 1 minute and was incubated at 4°C to allow for further coagulation. After coagulating for at least 4 hours, the blood was centrifuged at 4°C for 5 minutes, and the top layer of serum was transferred to a new Eppendorf tube. The serum was centrifuged at 4°C for 5 minutes to remove all clotted red blood cells. The pure serum was then transferred to a new Eppendorf tube and stored at -20°C for future analysis.

#### Isolation and Extraction of Parasites from Blood Culture

*P. falciparum* strain 3D7 asexual parasites and gametocytes were produced in culture and harvested by centrifugation. The parasites were purified using a discontinuous Accudenz [Accurate Chemical and Scientific Corporation; Westbury, NY] gradient. The Accudenz solution (0.3 mM CaCl<sub>2</sub>, 0.3 mM Na<sub>2</sub>EDTA, 5 mM Tris-HCl at pH 7.5, 3 mM KCl, 27.6% Accudenz) was diluted with sterile 1X PBS to 18% Accudenz for the purification of asexual parasites and early stage gametocytes (stages I-III) and was diluted to 16% Accudenz for the purification of late stage gametocytes (stages IV and V). The cell culture suspension was underlaid with the Accudenz solution and centrifuged at 3000 rpm for 20 minutes. The parasites were harvested from the parasitized cell layer containing schizonts and gametocytes. Some preparations of gametocytes were then treated with 0.015% saponin to lyse the red blood cells and isolate parasites from their host cells. To isolate gametes, mature stage V gametocytes were harvested by

centrifugation, induced to undergo gametogenesis by resuspension in human serum and 10  $\mu$ M xanthurenic acid, and incubated for one hour at room temperature. Accudenz was diluted to 11% for the purification of gametes, and the cell culture was underlaid with the diluted Accudenz solution and centrifuged at 3000 rpm for 20 minutes. The gametes were collected from the top of the Accudenz layer.

The collected cells were used in either immunofluorescence assays (IFA) or for protein extraction for western blots. For use in IFA, the cells were washed three times in 1X PBS and placed on a glass coverslip. Protein extract from asexual parasites, gametocytes, and gametes was obtained by incubating approximately  $5 \times 10^7$  parasitized cells with 50  $\mu$ l NETT solution (0.15 M NaCl, 5 mM EDTA, 50 mM Tris at pH 7.4, 1.0% Triton X-100, 0.05% Na azide) and 0.5  $\mu$ l each of 1 mM leupeptin, pepstatin, and aprotinin protease inhibitors. This reaction mixture was centrifuged at 13000 rpm at 4°C for 20 minutes, and the supernatant containing the protein extract was used for analysis by western blot.

For a timecourse experiment, seven large flasks of 3D7 asexual parasites were set up at the same time with an equal level of low parasitemia (0.2%). These flasks were allowed to grow and proceed through gametocytogenesis. The flasks were fed every day with 25 ml RPMI media throughout the experiment and were monitored by blood smears. When the flasks were at a high level of asexual parasites (approximately 10-15%), the first flask was collected using the Accudenz gradient method described above. After this collection, all flasks were treated with 1.25 ml 1 M N-acetyl glucosamine (NAG) for three days to prevent the future invasion of RBCs by merozoites. This NAG treatment allowed for a loose synchronization of the parasites throughout the rest of the timecourse

by eliminating most asexual parasites. Another flask was collected in the same way every other day, and on the final day of the experiment, two flasks were collected, one containing stage V gametocytes and one that was induced to undergo gametogenesis using the method described previously. IFA slides were also made on the same days that the parasite flasks were collected.

#### Characterization of Antiserum

Western blots were used to determine the size of protein(s) recognized by the antibodies generated in mice against the injected recombinant protein. Western blots were additionally used to determine which parasite life cycle stages contained the protein of interest. To perform a western blot, the protein extract from the asexual parasites, gametocytes, and/or gametes was size-fractionated on a 4-20% Tris-glycine polyacrylamide gel [Invitrogen] in running buffer (25 mM Tris-base, 200 mM glycine, 10% SDS) for 95 minutes at 126 V. The gel was then transferred to a nitrocellulose membrane of pore size 0.2  $\mu\text{m}$  [OPTITRAN BA-S 83, Whatman®; Dassel, Germany] by electrophoresis at 26 V for 3 hours in chilled transfer buffer (25 mM Tris-base, 25 mM glycine, 10% methanol).

The following morning, the membrane was stained with Ponceau S to evaluate protein transfer to the membrane and then blocked with 5% dry milk in 1X PBS for 15 minutes at room temperature to prevent non-specific antibody binding. The membrane was then incubated at 4°C overnight in the primary antibody under analysis in the mice serum, typically at a 1:500 dilution in 1X PBS. The following day, the membrane was washed 3 times with 1X PBS and then incubated for 4-6 hours at 4°C in the secondary antibody, Anti-Mouse IgG (Fc specific) – Alkaline Phosphatase produced in goat [Sigma

Chemical Company], at a 1:2000 dilution in 1X PBS. The membrane was then washed three times in 1X PBS before it was stained with 16.5  $\mu$ l BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) [Promega] and 33  $\mu$ l NBT (nitroblue tetrazolium chloride) [Promega] in 5 ml alkaline phosphatase buffer (100 mM NaCl, 5 mM MgCl<sub>2</sub>, 100 mM Tris-base at pH 9.5). The location of the bound antibodies was visualized by the formation of a grey-blue precipitate on the membrane.

Immunofluorescence assays (IFA) were used to determine the presence and location of the proteins of interest in different developmental stages of *P. falciparum* parasites. As mentioned previously, parasites either directly from culture or harvested using the Accudenz gradient were washed three times in 1X sterile PBS and applied to a dry coverslip [Fisher Scientific], pre-coated with 0.1% poly-l-lysine in 5% (w/v) sodium bicarbonate. After 30 minutes of incubation, the coverslips were washed three times in 1X sterile PBS. The cells were then fixed by incubation with 1% formaldehyde in sterile PBS for 10 minutes. 0.005% glutaraldehyde was added to the fixative solution for the study of soluble proteins (Spielmann et al. 2006). The fixative solution was removed, and the coverslips were stored in 1X PBS at 4°C overnight. The next day, the coverslips were incubated for 10 minutes in 50 mM NH<sub>4</sub>Cl to quench autofluorescence from free aldehyde groups. The coverslips were washed three times with 1X PBS and then incubated in 0.05% saponin for 20 minutes to permeabilize the red blood cells. Next, the coverslips were incubated in a blocking solution of 0.2% fishskin gelatin [Sigma Chemical Company] and 0.01% saponin for 30 minutes at 37°C. The coverslips were removed from the blocking solution and incubated with the primary antibody in the mice serum, typically at a 1:200 dilution in 0.01% saponin in sterile 1X PBS, for 1 hour at

room temperature. After the hour of incubation, the coverslips were washed three times with 0.01% saponin in sterile 1X PBS. The coverslips were then incubated with the secondary antibody, typically goat-anti-mouse IgG TRITC conjugate (Tetramethylrhodamine isothiocyanate) [Sigma Chemical Company] at a 1:200 dilution in 0.01% saponin in sterile 1X PBS, for 1 hour at room temperature. The coverslips were then washed three times with 0.01% saponin in sterile 1X PBS. The coverslips were mounted on glass slides with 0.3 µl Vectashield® mounting medium for fluorescence with DAPI [Vector Laboratories, Inc.; Burlingame, CA], and sealed to the slides with nail polish. The cells were then evaluated for fluorescence with a fluorescence microscope.

#### Immunoprecipitation

An immunoprecipitation was used to determine what proteins PFL2550w protein interacts with. The Pierce® Crosslink Immunoprecipitation Kit [Thermo Scientific; Waltham, MA] was used to perform the immunoprecipitation, per the kit protocol. First, a Pierce Spin Column was prepared, and the PFL2550w antibodies were coupled to the column resin. Another column was prepared in the same manner with anti-Maltose Binding Protein (MBP) antibodies bound to the resin to serve as a negative control, and all subsequent steps in the procedure were done to both columns. Disuccinimidyl suberate (DSS) was then used to crosslink the bound antibodies to the resin. Parasitized red blood cell lysate was then obtained using the instructions in “Protocol II: Lysis of Cell Suspension Cultures.” Two separate columns containing Pierce Control Resin were used to pre-clear the cell lysate; the pre-cleared lysate was then added to the columns with bound antibodies. The antigen in each column was eluted in five elutions of 25 µl Elution Buffer.

After collecting the eluates from the anti-PFL2550w antibody column in the Elution Buffer, the eluates were run on a 4-20% Tris-glycine polyacrylamide gel, and a western blot was performed to confirm that the eluates contained the PFL2550w protein. The eluates that were found to contain the highest concentrations of protein, measured by the intensity of the reactive bands in the western blot, were combined together. These combined elutes and the combined eluates obtained from the anti-MBP negative control column were run in two separate lanes for 5 minutes at 126 V on a fresh 4-20% Tris-glycine polyacrylamide gel and then stained with NOVEX® Colloidal Blue Staining Kit [Invitrogen]. The bands in the gel containing pure immunoprecipitation-eluted proteins from the PFL2550w antibody column and the MBP negative control column were stained blue by the staining kit. These bands were excised and sent for Mass Spectrometry analysis [Microchemistry and Proteomics Analysis Facility; Harvard University; Cambridge, MA] to determine what proteins were present in the eluate samples.

## CHAPTER III

### RESULTS

#### Purification and SDS-PAGE Analysis of PFL2550w Recombinant Protein

A PFL2550w recombinant protein construct was prepared to be used for the induction of antibody production in mice. The generated PFL2550w-MBP recombinant protein construct was confirmed to be correct by sequencing and restriction digestion analysis. The construct was expressed in *E. coli* and purified using an amylose resin column as described in the Materials and Methods. The recombinant protein fractions were eluted from the column with column buffer containing 10 mM maltose, and the eluates were characterized by size fractionation with a 4-20% polyacrylamide gel (Fig. 5). The upper band at 70 kDa is the partially degraded recombinant protein comprised of MBP and PFL2550w. The lower band at 42 kDa is likely MBP without the PFL2550w protein. The protein concentration appears to be the highest in elutions 1-4. Elution 2 of the recombinant protein was then used for injection into mice for antibody production.

#### PFL2550w Western Blot with Asexual and Gametocyte Extracts

To determine if the antibodies generated against the PFL2550w-MBP recombinant protein specifically recognized the parasite-produced PFL2550w, a western blot was performed with extracts of mixed 3D7 gametocytes and mixed 3D7 asexual parasites, and RBC protein extract was used as a negative control. *P. falciparum* asexuals and gametocytes were cultured *in vitro* and harvested using an Accudenz gradient. The

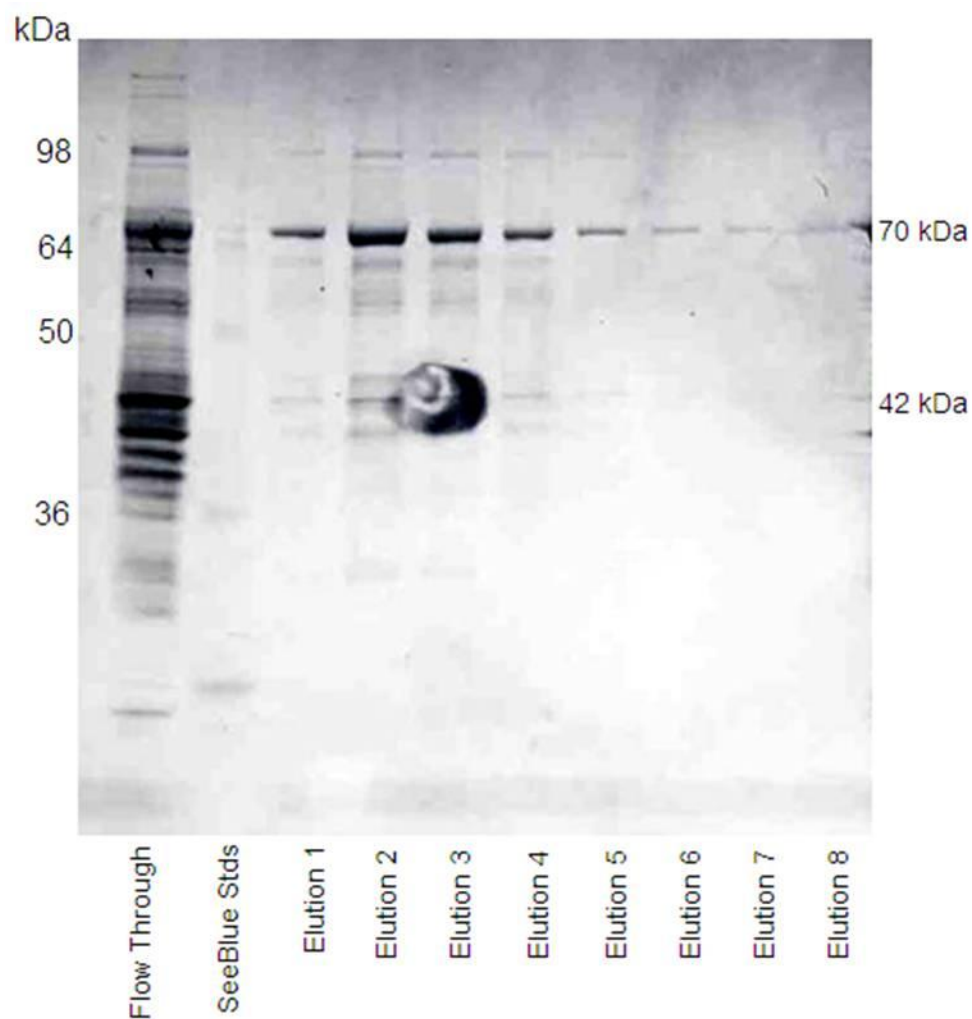


Figure 5. Amylose resin purification of PFL2550w recombinant protein. Aliquots of the material that did not bind to the column (Flow Through) and the material eluted from the column were run on a 4-20% polyacrylamide gel and stained with Coomassie blue.



proteins were extracted by NETT with protease inhibitors. Uninfected RBC were collected separately and proteins were extracted by NETT. The protein extracts were mixed with SDS-PAGE sample buffer and each divided into two tubes.  $\beta$ -Mercaptoethanol (BME) was added to one tube for each protein extract to further denature the proteins by breaking the disulfide bonds. The extracts were size fractionated on a 4-20% Tris-glycine polyacrylamide gel along with SeeBlue® molecular size standards, blotted on to a nitrocellulose membrane, and stained with Ponceau S to confirm transfer. The membrane was probed with the pooled blood serum (diluted 1:200 in 1X PBS) obtained from mice that had been previously injected with the PFL2550w recombinant protein. Figure 6A displays the Ponceau S staining of the nitrocellulose paper prior to antibody incubation, confirming that there was adequate transfer of the protein to the membrane. In the stained western blot, a distinct band is observed in the 3D7 gametocyte lanes (Fig. 6B). The size of the band (55.0 kDa) was interpolated from a semi-log plot prepared using the SeeBlue standard migration distances and sizes and was found to be consistent with the predicted gene product size, 55.68 kDa. The 55.0 kDa band appears identical in gametocyte lanes with and without BME, signifying that the addition of BME is not necessary for the study of this protein. There are no visible bands in the negative control RBC lanes, showing the parasite specificity of the antibodies, or the asexual parasite lanes, suggesting that this protein is not expressed in the asexual parasite life cycle stages.

#### PFL2550w Western Blots with Saponin-treated Gametocyte Extract

Next, western blots were conducted to test for the presence of the PFL2550w protein in the parasitized RBC. As PFL2550w is predicted to contain a secretory signal

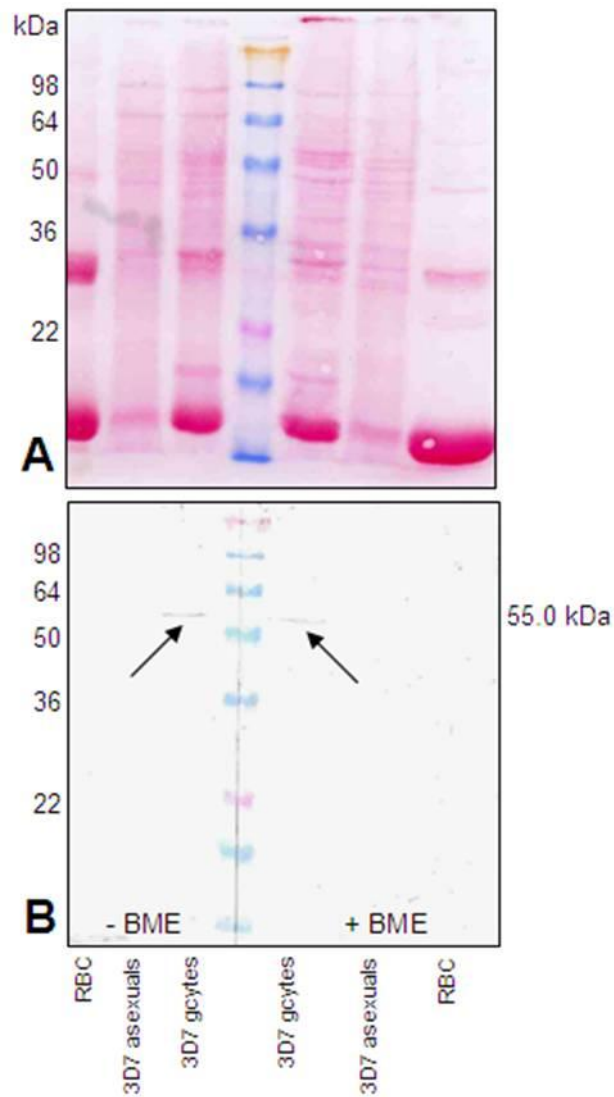


Figure 6. PFL2550w western blot of asexual and gametocyte extract, with and without BME. Transferred protein was detected by Ponceau S stain (A) and then probed with anti-PFL2550w antiserum (B). The parasite life cycle stages are indicated for each lane.

sequence and an erythrocyte export domain, it was hypothesized that the PFL2550w is exported from the parasite to the host RBC. To test this, a mixed culture of 3D7 gametocytes was harvested from the parasite layer on an 18% Accudenz gradient. Half of the collected parasites were transferred to a separate tube and incubated with 0.01% saponin for 5 minutes to lyse the RBC. Saponin treatment leads to hemolysis as saponin molecules complex with cholesterol to form pores in cell membrane bilayers. Protein was extracted from the saponin-treated and the non-saponin-treated parasites as described previously. This same procedure was also done to obtain protein extracts from gametocytes from another cultured strain of parasites, n230. These extracts were run on 4-20% Tris-glycine polyacrylamide gels, along with RBC and asexual protein extracts. The gels were then blotted on to nitrocellulose membranes that were stained with Ponceau S to confirm transfer and probed with the serum containing anti-PFL2550w antibodies.

Figures 7A and 7C displays the Ponceau S staining of the nitrocellulose papers prior to antibody incubation, confirming that there was adequate transfer of the protein to the membrane. The Ponceau S staining also helped to confirm that a comparable amount of parasite protein was added to each lane on the gel. The dark bands at 10 and 34 kDa that are seen in the extracts not treated with saponin are red blood cell proteins. Figure 7B displays the western blot conducted with 3D7 gametocytes. The 55.0 kDa band is again seen in the lane containing protein from gametocytes extracted from the Accudenz gradient; however no band is seen in the protein extract from gametocytes treated with saponin. Thus, when the host RBC is solubilized, the remaining intact gametocytes do not contain PFL2550w protein. This suggests that the PFL2550w protein is exported to the

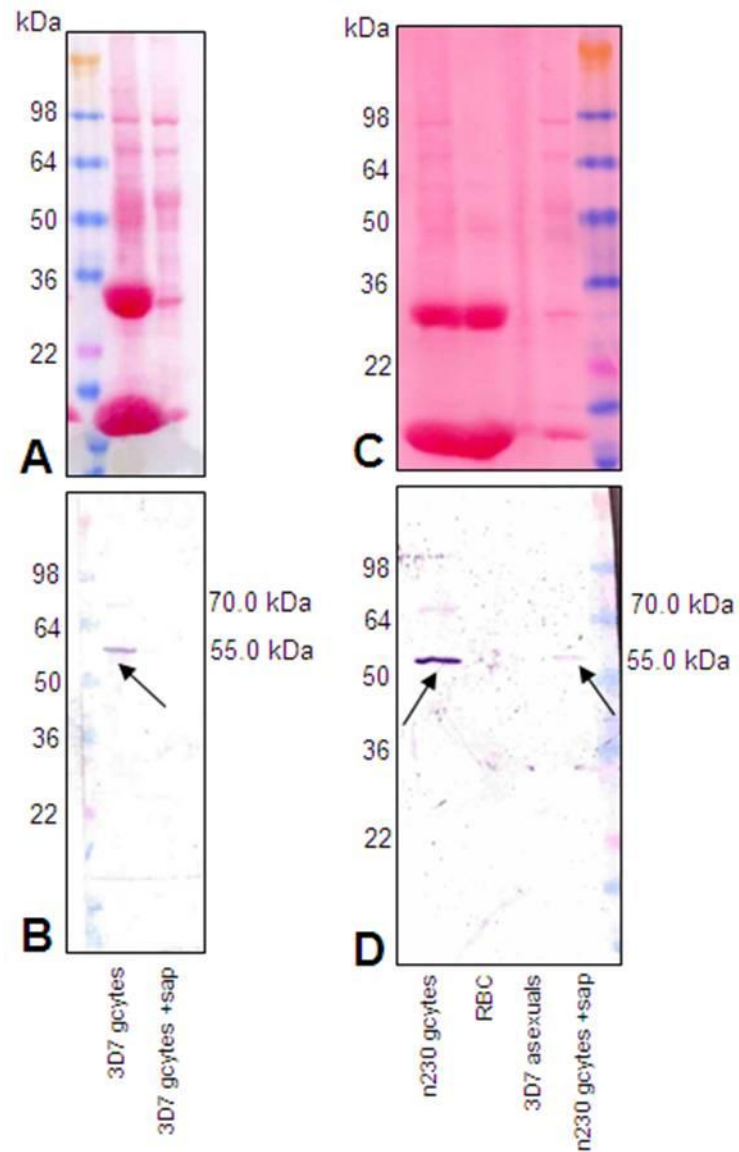


Figure 7. PFL2550w western blot of extracts from gametocytes treated without and with saponin (+sap). Transferred protein was detected by Ponceau S stain (A, C) and then probed with anti-PFL2550w antiserum (B, D). The parasite life cycle stages are indicated for each lane. Western blot on left was conducted with 3D7 strain gametocytes (A, B) and western blot on right was conducted with n230 strain gametocytes (C, D).

parasite's host RBC and consequently removed when the RBC is solubilized. The same pattern is observed in the western blot conducted with the n230 gametocytes (Fig. 7D). There is a dark band at 55.0 kDa corresponding to the PFL2550w protein in the protein extract from parasites collected from the Accudenz gradient. In the n230 gametocytes treated with saponin, there is a very faint band observed in that lane at the same size of 55.0 kDa, though the level of PFL2550w protein in this sample is clearly significantly reduced. The results are again consistent with the PFL2550w protein being exported to the host red blood cell *in vivo*, as removing the RBC from the gametocytes also removes the PFL2550w protein. In both western blots, a faint band is also seen in the Accudenz-only gametocyte lanes at 70.0 kDa. At this point, the composition of the 70.0 kDa band recognized by the PFL2550w antibodies remains unclear, but this larger band was a consistent finding. The band may be present due to some protein interaction with PFL2550w or a post-translational modification of PFL2550w protein.

#### PFL2550w Western Blot with Timecourse Parasite Extracts

To determine the protein expression of PFL2550w throughout gametocytogenesis, seven flasks of 3D7 asexual parasites were set up containing a low parasitemia (0.2%). Over the course of the following two weeks, the parasite cultures grew and proceeded to mature stage V gametocytes. When the flasks reached 10-15% asexual parasitemia, parasites were harvested from the first flask using the Accudenz gradient method described in the Materials and Methods (day 6). Another flask was collected in the same way every other day. Giemsa-stained smears were made each day that parasites were collected to determine the predominant stages of parasites in that days flask. On the final day of the experiment (day 16), two flasks were collected, one containing stage V

gametocytes and one that was induced to undergo gametogenesis. The protein from the seven tubes of collected parasites was extracted using NETT. The protein extract from approximately  $2.5 \times 10^6$  parasites per lane was size fractionated on a 4-20% Tris-glycine polyacrylamide gel and blotted on to a nitrocellulose membrane, which was stained with Ponceau S to confirm transfer. The blot was probed with the anti-PFL2550w serum.

Figure 8A displays the Ponceau S staining of the nitrocellulose membrane, confirming both adequate protein transfer and that a similar amount of protein extract was run on each lane of the gel. The predominant parasite stages from each collected flask are listed at the bottom of Figure 8. The western blot shows that the 55.0 kDa band corresponding with the *P. falciparum* PFL2550w protein is the most prominent band, and the 70.0 kDa band is again seen much more faintly (Fig. 8B). The highest levels of PFL2550w protein are observed in the gametocyte lanes with parasites at stages I & II and II & III (3D7 gcytes I&II, 3D7 gcytes II&III), as the stained bands at 55.0 kDa are the darkest in those lanes, showing the strongest reactivity of the antibodies with the PFL2550w protein. The lanes containing other gametocyte stages (stages I, III & IV, and IV & V) also have some reactivity with the PFL2550w antibodies, though the bands are fainter, signifying a lower amount of PFL2550w protein. The 3D7 asexual and 3D7 gamete lanes have almost no reactivity at 55.0 kDa and no visible reactivity with the higher protein at 70.0 kDa. This figure clearly depicts the overall pattern of protein expression for the PFL2550w protein. There is little to no protein expressed in asexual parasites. As parasites enter gametocytogenesis, the amount of PFL2550w protein increases from stage I to stage II. Following stage II, there is a decrease in protein level

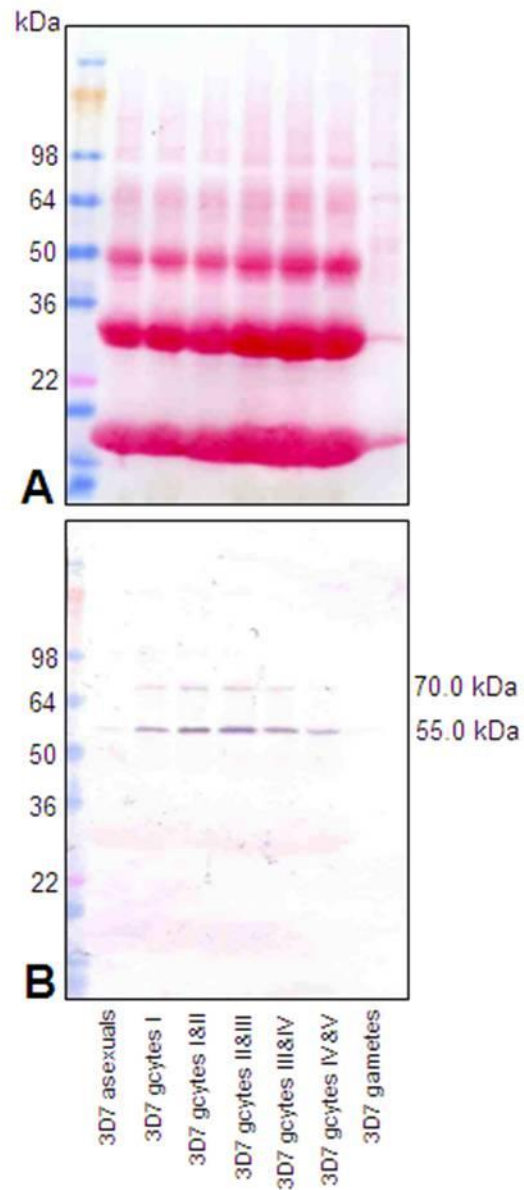


Figure 8. PFL2550w western blot of extracts from parasite timecourse. Transferred protein was detected by Ponceau S stain (A) and then probed with anti-PFL2550w antibodies (B). The predominant parasite life cycle stages are indicated for each lane.

through the remaining stages of gametocytogenesis, and no protein is observed at the gamete stage.

#### PFL2550w Immunofluorescence Assays

The western blot experiments successfully confirmed the specificity of the generated antibodies and identified the general protein expression pattern of PFL2550w and the protein's location in the infected erythrocyte. Next, the anti-PFL2550w antibodies were tested for their ability to recognize parasite-produced PFL2550w protein by immunofluorescence assays (IFA). These assays were also used to analyze the subcellular localization of the PFL2550w protein and the protein's stage specific expression. The parasites were fixed on coverslips with 1% formaldehyde, prior to incubation with antiserum, however variable reactivity was observed. The addition of 0.005% glutaraldehyde to the fixative solution improved both the clarity and the consistency of the results (Spielmann et al. 2006). IFA slides and analyses were done concurrently with the timecourse western blot experiment described previously, to obtain a picture of protein expression throughout parasite development (Fig. 9). Only a small subpopulation of ring and trophozoite stage parasites reacted with the anti-PFL2550w antibody. In contrast, a majority of stage I and II gametocytes were positive. Interestingly, the epi-fluorescence was located almost exclusively in the host red blood cell in gametocytes and not in the parasite itself. Many stage III gametocytes were also positive but had a rim fluorescence pattern (Fig. 9, stage III panel). For all stage IV and V gametocytes and gametes, the amount of fluorescence observed was identical to that of the negative control for those stages and was considered background fluorescence. The protein expression pattern and levels observed in the IFA analyses correlated well with



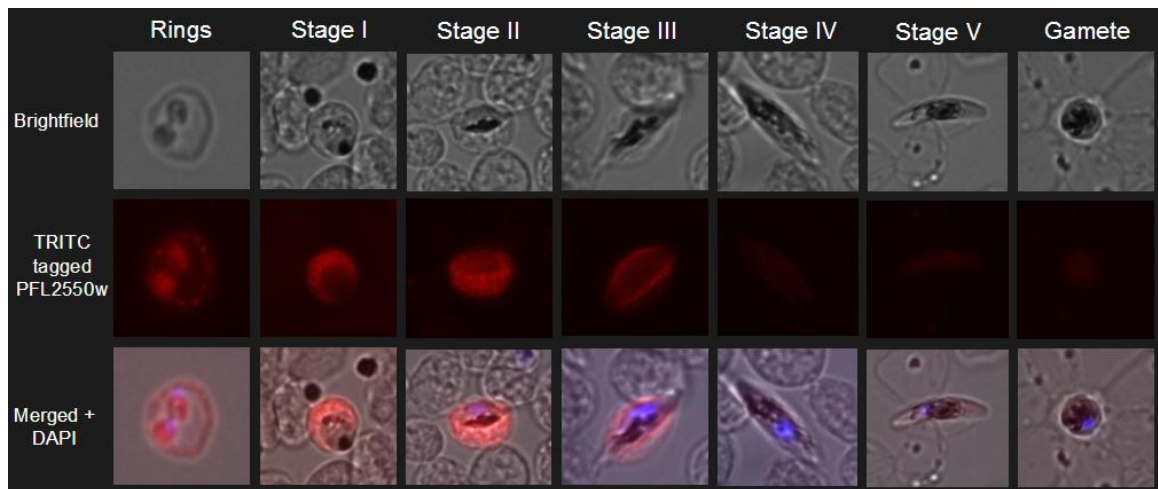


Figure 9. IFA analysis of PFL2550w protein expression and localization throughout parasite development. The images in each column show the same microscopic field. The top row shows the brightfield images, the middle row shows the localization of the PFL2550w protein tagged with TRITC secondary antibody illuminated by UV light, and the bottom row shows the merged images of brightfield, TRITC-tagged PFL2550w, and the fluorescent nucleic acid stain, DAPI.

the protein expression pattern seen in the western blot analyses. The fluorescence of some but not all asexual stage parasites may be due to PFL2550w protein expression only in asexual parasites committed to gametocytogenesis.

#### Colocalization IFA of PFL2550w and Pfs16

After confirming the reactivity of the PFL2550w antibodies with parasites in the IFA analysis described previously, colocalization IFA experiments were done to observe the localization of PFL2550w protein in relation to other previously characterized *P. falciparum* proteins. Colocalization experiments were done by fixing parasites on coverslips, probing the parasites with anti-PFL2550w antiserum from mice as well as primary antibodies against another *P. falciparum* protein generated in rabbit, and then using anti-mouse IgG TRITC and anti-rabbit IgG FITC (Fluorescein isothiocyanate) as secondary antibodies. Figure 10 shows the colocalization of the PFL2550w antibodies and antibodies against sexual stage-specific *P. falciparum* protein Pfs16 in stage I and II gametocytes. The Pfs16 protein, tagged with FITC (green), is located in the parasitophorous vacuole whereas PFL2550w, tagged with TRITC (red), is predominately located in the host red blood cell.

#### Counts of PFL2550w and Pfs16 Expressing Parasites

An IFA experiment was done with the colocalization of PFL2550w and Pfs16 antibodies on Accudenz gradient purified schizonts (“schizonts”), trophozoites and early stage gametocytes (“non-schizonts”) to determine if the two antibodies were reactive and specific to the same parasites and parasitized red blood cells. Stage I gametocytes and asexual trophozoites were classified as “non-schizonts” as they are indistinguishable by microscopy. As the parasitized red blood cells used in this experiment were purified by

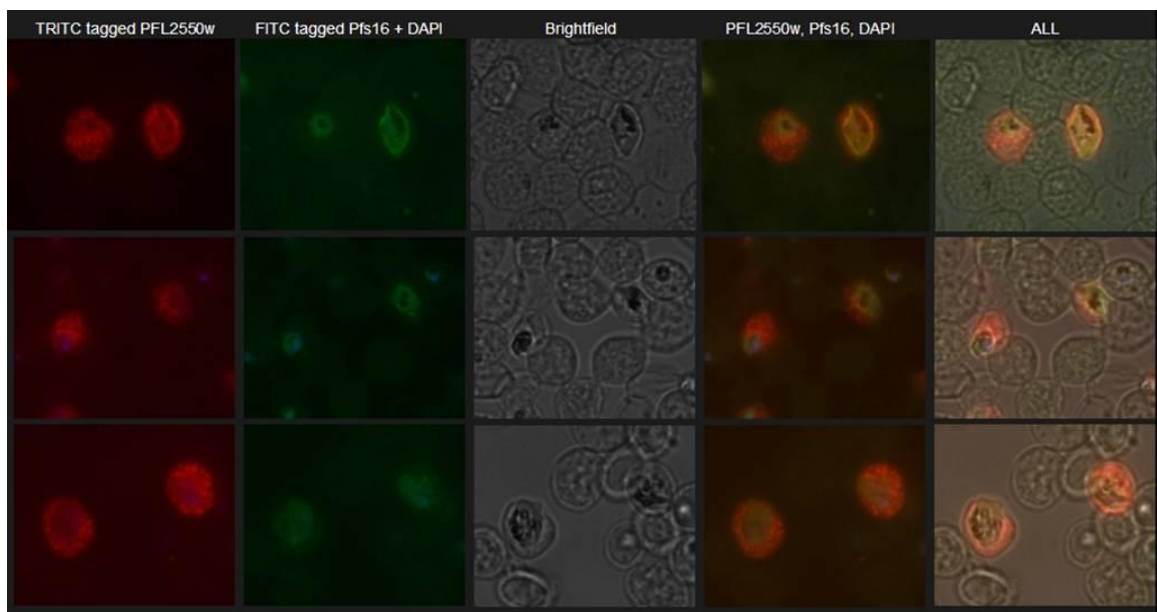


Figure 10. Colocalization IFA analysis of PFL2550w and Pfs16 antibodies on stage I and II gametocytes. Secondary antibody for PFL2550w was anti-mouse IgG TRITC (red) and secondary antibody for Pfs16 was anti-rabbit IgG FITC (green).

an Accudenz gradient, no ring stage parasites were present on the IFA slides. In previous IFA experiments when analyzing PFL2550w expression in asexual ring stage parasite cultures at low parasitemia (<5%), no positive PFL2550w fluorescence was observed on a consistent basis. PFL2550w positive parasites have been observed regularly only in asexual cultures at high parasitemia (>10%) that were beginning to crash and transition to gametocytogenesis, as well as in gametocyte-only cultures. It is unclear at what point in development the PFL2550w protein begins to be expressed, but in our IFA experiments, PFL2550w positive parasites were rarely seen in asexual cultures. Thus, a high parasitemia culture of schizonts and early gametocytes purified by Accudenz gradient was used for this counting experiment to ensure the presence of many PFL2550w positive parasites on the slide for more accurate counts (Fig. 11).

Two levels of fluorescence were observed for both antibodies, and bright and faint fluorescent tagging were counted separately. While the numbers of brightly and faintly fluorescing parasites appears to be similar for the two antibodies, the same parasites were not always bright for both antibodies or faint for both antibodies (Fig. 12). Another observation from this experiment was that the majority of the faintly-tagged parasites for the PFL2550w antibodies appeared to be schizonts. Most brightly-tagged parasites with anti-PFL2550w were identified visually as gametocytes. Many of the parasites faintly positive for Pfs16 were identified as schizonts.

#### Localization IFA with SBP1

An IFA analysis was conducted to determine if PFL2550w localized to the Maurer's clefts, flat lamellar parasite-generated organelles located in the infected erythrocyte cytoplasm. Skeleton binding protein 1 (SBP1) is a *P. falciparum* protein

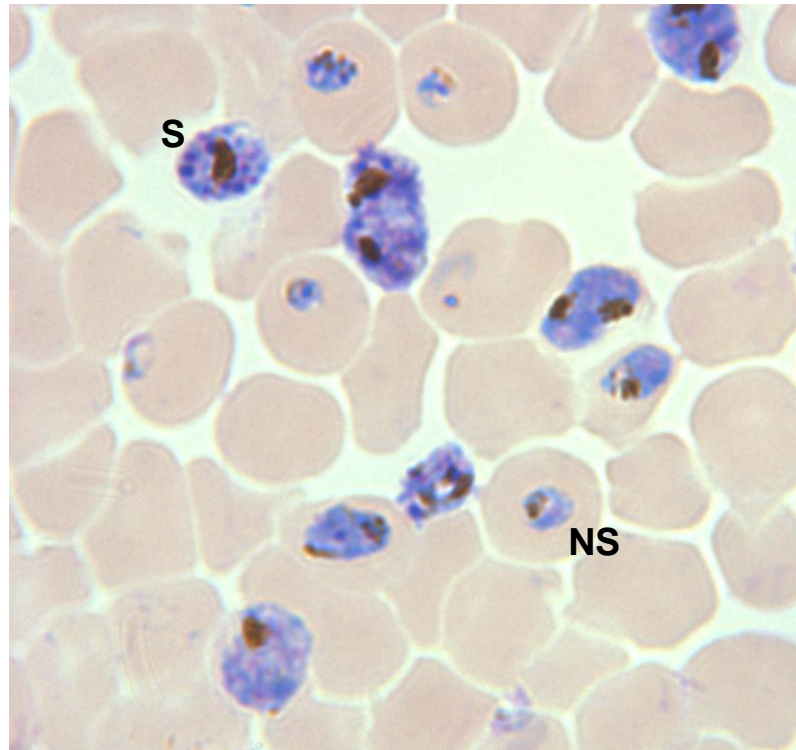


Figure 11. Giemsa-stained smear of Accudenz purified schizonts (S) and “non-schizonts” (NS) – trophozoites and early gametocytes. These parasites were used for the IFA count experiment.

| Total Parasites<br>per ~800 RBCs |                  | PFL2550w<br>positive |              | Pfs16 positive |              |
|----------------------------------|------------------|----------------------|--------------|----------------|--------------|
| <i>Non-Schizonts</i>             | <i>Schizonts</i> | <i>Bright</i>        | <i>Faint</i> | <i>Bright</i>  | <i>Faint</i> |
| 25                               | 51               | 22                   | 37           | 18             | 55           |
| 30                               | 43               | 26                   | 30           | 21             | 36           |
| 29                               | 44               | 26                   | 32           | 23             | 38           |
| % of total parasites:            |                  | 33.3%                | 44.6%        | 27.9%          | 58.1%        |

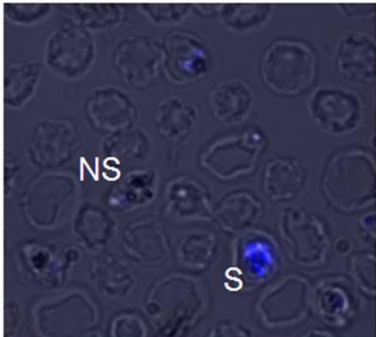
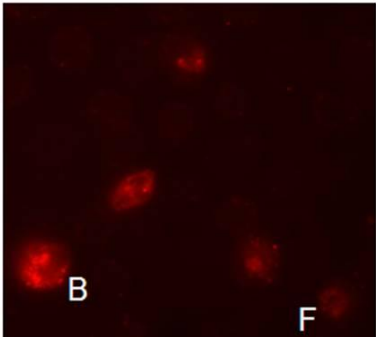
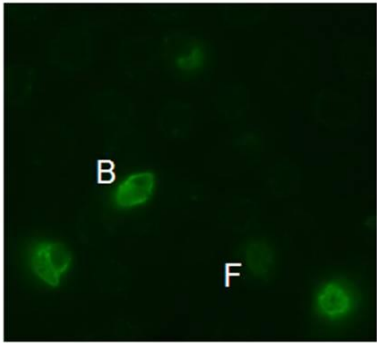




Figure 12. Counts of total parasites, PFL2550w positive parasites, and Pfs16 positive parasites per 800 red blood cells. Representative pictures of Non-Schizonts (NS), Schizonts (S), Bright fluorescence (B), and Faint fluorescence (F) below table. The table lists the total number of parasites (non-schizonts and schizonts) present in a microscope field, the number of PFL2550w positive parasites (bright and faint fluorescence), and the number of Pfs16 positive parasites (bright and faint fluorescence). Each row of the table corresponds with the numbers of parasites counted per approximately 800 RBCs. All parasites counted in the first two columns were totaled to 222 parasites, and the percentage of brightly- and faintly-tagged parasites for each antibody was calculated.

known to reside in the Mauer's clefts. To determine if PFL2550w protein also localizes to the Mauer's clefts, anti-SBP1 antibodies (generated in mice) were used for an IFA analysis on stage I and II gametocytes (Blisnick et al. 2000). As previously seen in the literature, a punctate pattern localized to the periphery of the RBC was observed for SBP1 protein (data not shown). Our experiments show that the PFL2550w protein was localized throughout the entire RBC cytoplasm and did not appear to be localized exclusively to the Mauer's clefts with SBP1.

#### PFL2550w Immunoprecipitation

An immunoprecipitation experiment was conducted using PFL2550w antibodies to determine what proteins PFL2550w interacts with *in vivo*. PFL2550w antibodies were bound to beads in one immunoprecipitation column, while anti-MBP antibodies were bound to beads in a separate immunoprecipitation column to serve as a negative control, as MBP is not a *P. falciparum* protein. *P. falciparum* parasites were collected from blood cultures using an 18% Accudenz gradient and washed in sterile 1X PBS. The parasitized RBC were lysed, and the lysate was passed through the columns. Proteins bound to the columns were then eluted in five separate elutions each with 25  $\mu$ l buffer. A 1.5  $\mu$ l aliquot of each elution from the PFL2550w column, as well as flow through (FT) solutions from previous steps in the experiment, were analyzed by western blot with anti-PFL2550w antibodies to confirm that the elutions contained PFL2550w protein (Fig. 13). No PFL2550w protein was detected in the FT. The PFL2550w antibodies did exhibit reactivity with the elution samples, primarily with elutions 1 and 2. The size of the visualized bands appears to be slightly larger than the typical size of PFL2550w, 55.0 kDa. This apparent size difference may be due to a slight dissimilarity of how the protein

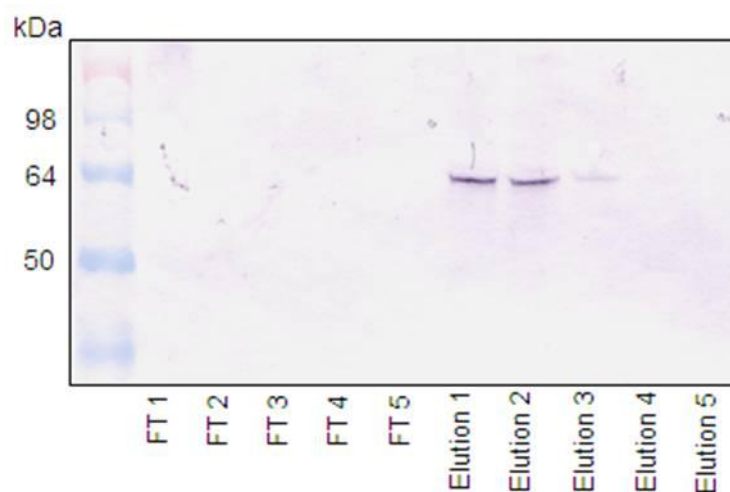


Figure 13. Western blot of PFL2550w immunoprecipitation. A western blot of aliquots from different steps through the immunoprecipitation was probed with anti-PFL2550w antiserum. FT = flow through.



eluates, containing a relatively small amount of protein, ran on the polyacrylamide gel.

The western blot confirmed that the immunoprecipitation column was successful in binding the PFL2550w protein antigen and that the PFL2550w protein was successfully eluted from the column with elution buffer.

The PFL2550w eluates 1 and 2 were combined, as were MBP negative control eluates 1 and 2, and run in separate lanes for 5 minutes at 126 V on a 4-20% polyacrylamide gel. The gel was stained with NOVEX® Colloidal Blue Staining Kit [Invitrogen] to visualize the protein bands (data not shown). The stained blue bands containing the pure immunoprecipitation eluates (sample 3), as well as two small gel pieces directly above each band (samples 1 and 2) were excised and sent for Mass Spectrometry analysis [Microchemistry and Proteomics Analysis Facility; Harvard University]. The Mass Spectrometry results were analyzed and sorted to remove all proteins identified in both the MBP negative control samples and the PFL2550w samples.

Thirteen *Plasmodium* specific genes/proteins were identified that were exclusive to the PFL2550w column eluates, including PFL2550w protein. Table 1 lists these thirteen proteins and their relative peptide concentrations in the MBP negative control gel samples (MBP1, 2, and 3) and the PFL2550w gel samples (2550 1, 2, and 3). The “Total 2550” column displays the total relative peptide concentration for each of the thirteen proteins identified. The Log(ratio) column represents the ratio of the protein concentration found in the PFL2550w samples compared to the amount found in the negative control (MBP) samples. Peptides corresponding to PFL2550w were specifically immunoprecipitated by PFL2550w antibodies. No other peptides were found in such high amounts. For the thirteen *Plasmodium*-specific proteins listed in Table 1, the literature

Table 1. Immunoprecipitation-identified proteins exclusive to PFL2550w column eluates.

|               | Control samples |      |      |        |        |        |       |           |            |            |
|---------------|-----------------|------|------|--------|--------|--------|-------|-----------|------------|------------|
| Gene/Protein  | MBP1            | MBP2 | MBP3 | 2550 1 | 2550 2 | 2550 3 | Total | Total MBP | Total 2550 | Log(ratio) |
| PFL2550w      | .               | .    | .    | .      | 36     | 37     | 73    |           | 73         | 9.5        |
| PF14_0632     | .               | .    | .    | .      | 2      | .      | 2     |           | 2          | 4.3        |
| PFC1020c      | .               | .    | .    | .      | 2      | .      | 2     |           | 2          | 4.3        |
| PFC0110w      | .               | .    | .    | .      | 2      |        | 2     |           | 2          | 4.3        |
| PF11_0175     | .               | .    | .    | .      | 2      |        | 2     |           | 2          | 4.3        |
| PFL2505c      | .               | .    | .    | .      | 2      |        | 2     |           | 2          | 4.3        |
| PF14_0636     | .               | .    | .    | .      | 2      |        | 2     |           | 2          | 4.3        |
| PB000529.03.0 | .               | .    | .    | .      | 2      | .      | 2     |           | 2          | 4.3        |
| PF13_0316     | .               | .    | .    | .      | 1      | .      | 1     |           | 1          | 3.3        |
| PFE0845c      | .               | .    | .    | .      | 1      | .      | 1     |           | 1          | 3.3        |
| PFE0040c      | .               | .    | .    | .      | 1      |        | 1     |           | 1          | 3.3        |
| PF14_0201     | .               | .    | .    | .      | .      | 1      | 1     |           | 1          | 3.3        |
| PF07_0007     | .               | .    | .    | .      | 1      |        | 1     |           | 1          | 3.3        |

was referred to in order to determine if any of the proteins identified were likely found in the same cellular location as PFL2550w. From the list, PF11\_0175 and PFE0040c were selected for further study due to their cellular locations, predicted functions, and mRNA expression profiles. PF11\_0175 codes for the HSP101 protein that was recently characterized as being a part of a novel protein export machine in malaria parasites (de Koning-Ward et al. 2009). PFE0040c codes for the PfEMP2 protein, a protein that is exported to the host erythrocyte and accumulates under the plasma membrane underneath knobs of infected erythrocytes. The mRNA expression profiles of these genes were isolated from microarray data and compared to the expression profile of PFL2550w (Fig. 14). All three genes show varied expression patterns throughout the asexual life cycle stages. Interestingly, the mRNA for all three genes shows a peak in expression at the onset of gametocytogenesis in gametocyte-producing NF54 strain parasites (NF54 Day 1, NF54 Day 2, etc.), and a steady decline in expression throughout the rest of NF54 gametocytogenesis.

#### Colocalization IFA of PFL2550w and HSP101

After being identified in the immunoprecipitation experiment, antibodies generated in rabbit against HSP101 were obtained from Brendan S. Crabb [Burnet Institute; Melbourne, Australia] and used for a colocalization IFA experiment with PFL2550w antibodies. Figure 15 shows the colocalization of PFL2550w antibodies with HSP101 antibodies. The HSP101 protein appears to be present throughout the parasitophorous vacuole, the location of the novel export machine, while PFL2550w appears to be localized primarily to the host erythrocyte. Not all parasites positive for PFL2550w were also positive for HSP101 and vice versa.

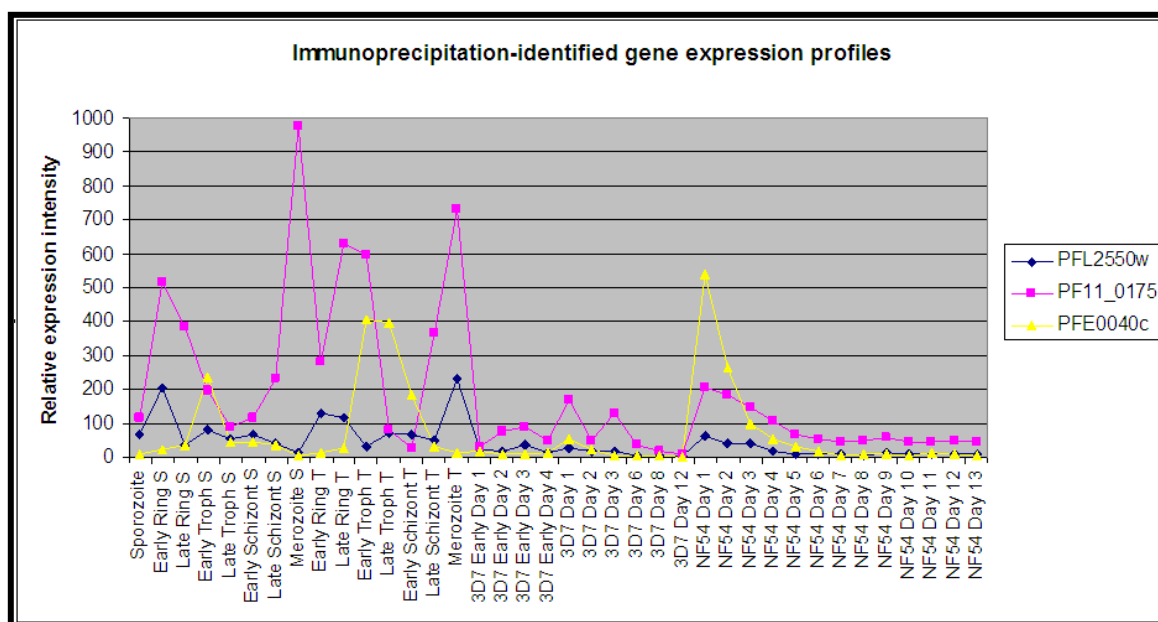


Figure 14. Expression profiles of immunoprecipitation-identified genes, PF11\_0175 and PFE0040c, with PFL2550w.

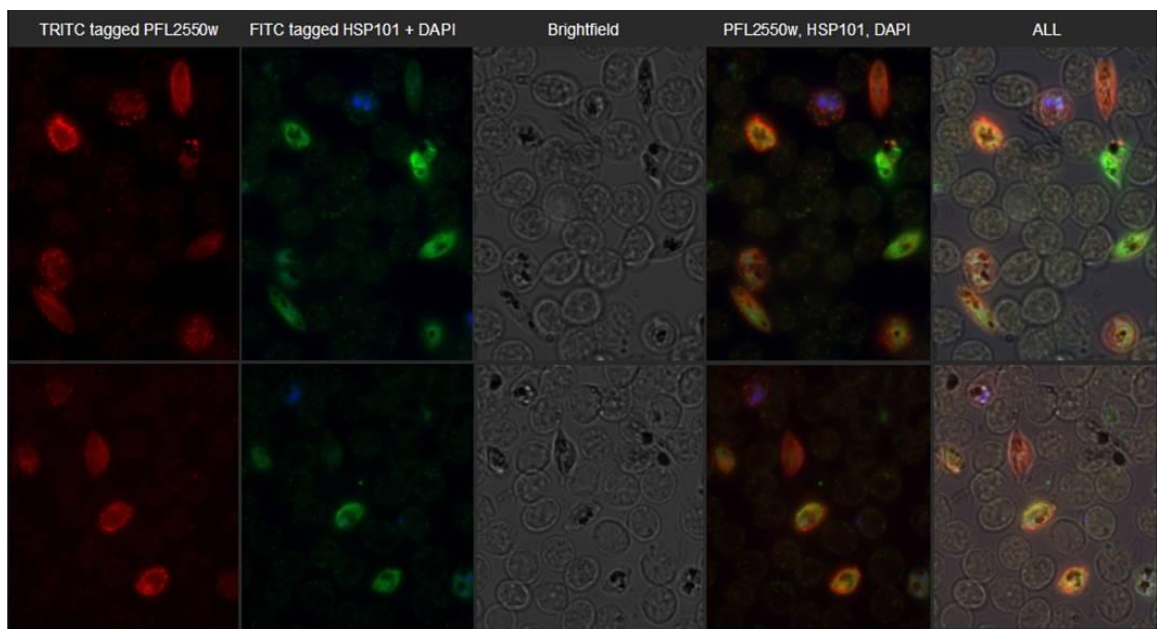


Figure 15. Colocalization IFA analysis of PFL2550w and HSP101 antibodies on mixed stage gametocytes. Secondary antibody for PFL2550w was anti-mouse IgG TRITC (red) and secondary antibody for HSP101 was anti-rabbit IgG FITC (green).

### DNA Construct Preparation for Parasite Transfections

DNA constructs for PFF0750w gene truncation and PFL2550w gene knockout and gene truncation were generated for transfection into parasites to analyze the phenotype of parasites lacking functional PFF0750w or PFL2550w protein. Prior to being used for parasite transfection, the purified plasmid DNA was analyzed by restriction digestion to confirm the correct insertion and size of the 3D7 *P. falciparum* DNA in the bacterial vector (Fig. 16, not pictured for PFF0750w). The PFL2550w gene truncation digested plasmid migrated in the gel as two bands (Fig. 16A). The upper band migrated between 6.759 and 8.144 kb, which is consistent with the expected size of the plasmid, 7.1 kb. The lower band migrated between 466 and 572 bp. The expected insert size in this construct is 634 bp. The slight size difference can be attributed to inconsistencies within the agarose gel used for analysis. The PFF0750w digested construct also migrated as two bands (data not shown). The upper band size was consistent with the expected plasmid size of 7.1 kb, and the lower band size was consistent with the expected insert size of 844 bp (data not shown).

For the PFL2550w gene knockout construct, two restriction digestions were done to confirm the presence of the F1 and F2 insert fragments in the pHHT-FCU vector (Fig. 16B). The upper band migrated between 9.023 and 22.845 kb. This is larger than the expected plasmid size, 7.6 kb. The size discrepancy may have resulted from the large amount of genetic material in the gel lane, causing the DNA to migrate a shorter distance than expected. The two lower bands correspond to the F1 and F2 fragments. The F1 insert migrated between 722 and 901 bp, which is very close to the expected size of the insert, 925 bp. The F2 insert migrated between 579 and 722 bp, which again is close to

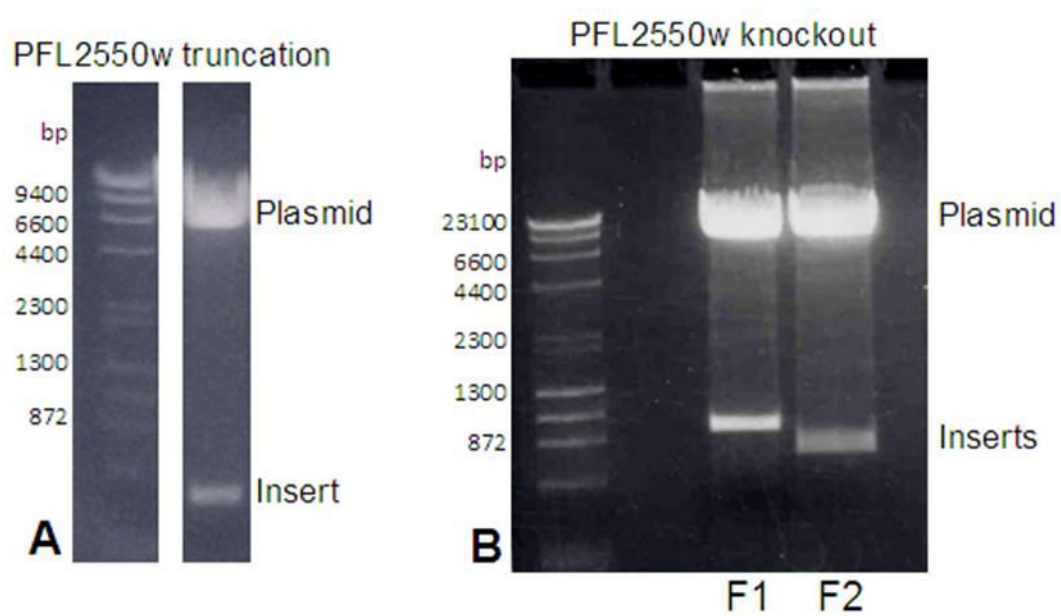


Figure 16. Restriction digest analysis of PFL2550w construct DNA for transfections.

the expected size of the insert, 760 bp. The slight discrepancies in size may again have resulted from inconsistencies in the agarose gel, or could also be accounted for by alterations in migration ability caused by the ion content of the restriction digestion buffers. This restriction digestion analysis, in addition to automated sequencing, confirmed that the constructs were correct and were able to be used for transfection into *P. falciparum* parasites in culture.

#### Transfection of PFF0750w Truncation Construct DNA into Parasites

The PFF0750w gene truncation construct was then transfected into parasites, as described previously, to interrupt the genomic PFF0750w gene and truncate the gene product. From two independent parasite transfections with the PFF0750w truncation construct, a mixed culture of parasites grew up in the selective media. DNA was extracted from each culture of PFF0750w gene truncation plasmid-transfected parasites, which were given the names J1 and J2. PCR was used to confirm that some parasites in each culture had properly integrated the construct into their genome. Four primers were prepared (Fig. 17) and used in four different two-primer combinations to selectively amplify episomal plasmid DNA (primers p65 and M13), wild type (WT) 3D7 *P. falciparum* DNA (primers p66 and p67), WT-plasmid crossover at the 3' end (primers p65 and p67), and WT-plasmid crossover at the 5' end (primers p66 and M13). These primers were used to amplify DNA extracted from J1 and J2 cultures as well as WT 3D7 *P. falciparum* as a positive control. No DNA template was used as a negative control. The 1014 bp band observed from amplification with primers p65 and M13, both primers that are located on the pDT.Tg23 vector, show that both J1 and J2 lines contain parasites with non-integrated, episomal plasmid. The 1014 bp band observed from amplification



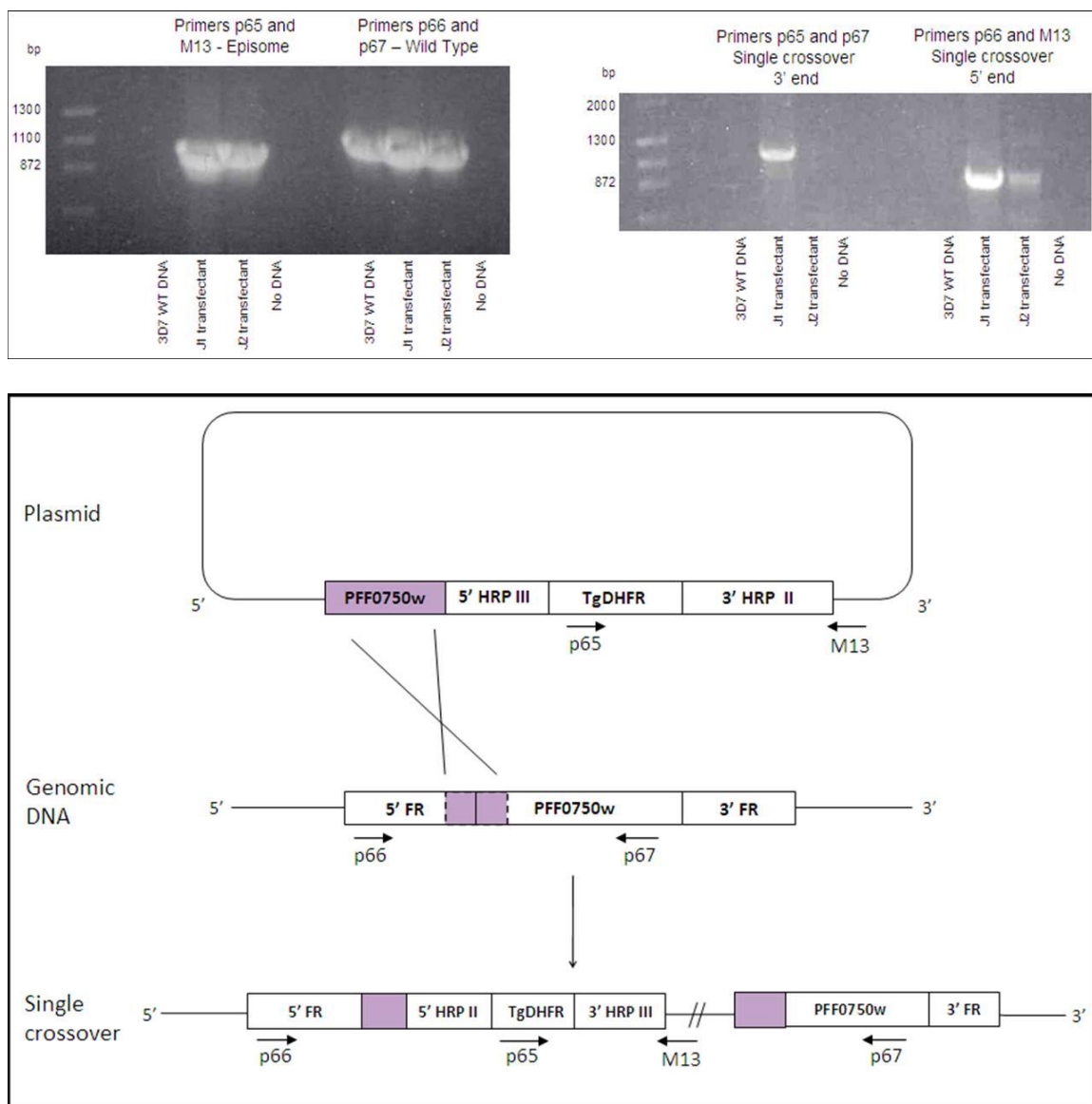


Figure 17. Integration PCR analysis of PFF0750w gene truncation transfectants J1 and J2. 3D7 WT DNA was used as a positive control and no DNA was used as a negative control in the PCR experiment.

with primers p66 and p67 that are both located on the WT *P. falciparum* DNA show the presence of WT parasites in both the J1 and J2 cultures, as well as in the 3D7 positive control, that have not integrated the construct into their genomes. The observed size of 1014 bp is smaller than the expected size of 1185 bp; the discrepancy may have resulted from inconsistencies in the agarose gel. The 1210 bp band observed from DNA amplification with primers p65 and p67, one primer in the bacterial vector and one in the WT DNA, show the presence of parasites that have integrated the construct DNA correctly by single crossover at the 3' end. Both the J1 and J2 cultures contain parasites with correct 3' integration, though the band observed in the J2 lane was unable to be seen in the gel picture. The 933 bp band observed from DNA amplification with primers p66 and M13, one primer in the bacterial vector and one in the WT DNA, show the presence of parasites that have integrated the construct DNA correctly by single crossover at the 5' end. The observed size of 933 bp is smaller than the expected size of 989 bp, again likely due to gel inconsistencies. Both the J1 and J2 cultures contain parasites with correct 5' integration.

The PFL2550w gene truncation and gene knockout construct have not yet been transfected into 3D7 *P. falciparum* parasites. When the transfections are performed and parasites have been collected, a similar procedure will be used to test for integration. Limited dilution cloning, to isolate a clonal population derived from a parasite with correctly integrated plasmid, has been attempted multiple times for both J1 and J2 cultures; however, all cloning attempts have been thus far unsuccessful.

#### Purification and SDS-PAGE Analysis of PFF0750w Recombinant Protein

A PFF0750w recombinant protein construct was prepared to be used for the

induction of antibody production in mice. The generated PFF0750w-MBP recombinant protein construct was confirmed to be correct by sequencing and restriction digestion analysis. The construct was expressed in *E. coli* and purified using an amylose resin column as described in the Materials and Methods. The recombinant protein fractions were eluted from the column with buffer containing 10 mM maltose, and the eluates were characterized using a 10% Tris-glycine gel (Fig. 18). As the fourth elution was determined to have the highest concentration of protein using the Bio-Rad Protein Assay, elution 4 was characterized by size fractionation at varying concentrations, 0.1 µg/µl, 0.01 µg/µl, and 0.001 µg/µl. MBP was also run as a control, with a band size of 43 kDa. The PFF0750w coding region is 2118 bp long; however, the recombinant protein construct was made with only 972 bp of the *P. falciparum* coding region for this gene. The size of the PFF0750w protein *in vivo* is approximately 83 kDa. As approximately half the coding region was used in this recombinant protein construct, it was expected that the size of the recombinant PFF0750w protein would be 38-42 kDa. Adding the size of MBP (43.5 kDa) would result in a recombinant protein product of size 81-85 kDa. There is a faint band at 80 kDa observed in the 0.1 µg/µl and 0.01 µg/µl lanes that is near the expected protein size. However, the strongest band observed in the gel for the PFF0750w recombinant protein has a size of 57 kDa, which is likely to be degraded protein. PFF0750w elution 4 recombinant protein was then prepared and used for injection into mice for antibody production, as described previously.

#### PFF0750w Western Blot with Gametocyte and Gamete Extracts

To determine if the antibodies generated against the PFF0750w recombinant protein were able to recognize the parasite-produced PFF0750w, western blots were

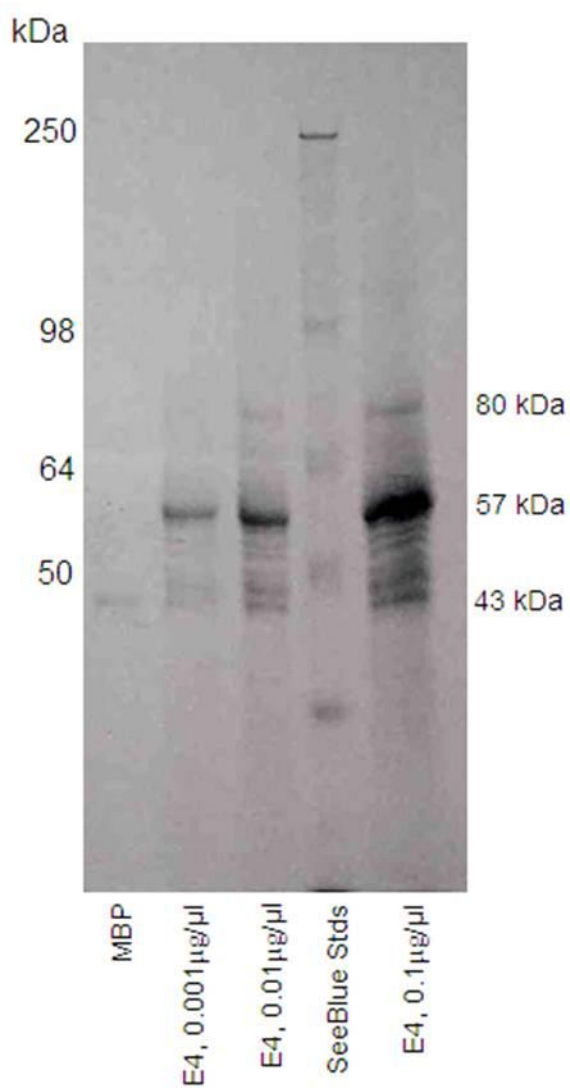


Figure 18. Amylose resin purification of PFF0750w recombinant protein (E4). Three concentrations of recombinant PFF0750w were run on a 10% polyacrylamide gel and then stained with Coomassie blue. MBP was run as a control.

done with 3D7 *P. falciparum* asexual, gametocyte, and gamete extracts. *P. falciparum* asexuals and gametocytes were cultured *in vitro*, were harvested using an Accudenz gradient, and the proteins were extracted by NETT with protease inhibitors. Parasites collected for these experiments were further purified by saponin treatment prior to protein extraction. The extracts were size fractionated on a 4-20% Tris-glycine polyacrylamide gel along with SeeBlue® standards and RBC extract as a negative control, transferred to a nitrocellulose membrane which was stained with Ponceau S to confirm transfer (not pictured), and probed with the serum obtained from the mice that had been injected with the PFF0750w recombinant protein.

No reactive bands were observed in the lanes containing negative control RBC extract or in lanes containing 3D7 asexual parasite protein extract. Once it was confirmed by multiple western blots that no reactive bands were present in asexual parasite protein extract, these extracts were not used in subsequent experiments. As seen in Figure 19A and C, a reactive band was observed in protein extract from 3D7 mixed late-stage gametocytes near the SeeBlue® standard at 98 kDa. This band was observed repeatedly but inconsistently in western blots with gametocyte extract. The reactive band at 98 kDa was observed in gametocyte extract in five western blots, and no band was observed in seven western blots. The band at 98 kDa is very faint and required a long staining time for adequate visibility. The actual size of PFF0750w *in vivo* is predicted to be 82.948 kDa. The size of the reactive band is thus larger than the actual protein size; however it is possible that the antibodies are specific for the PFF0750w protein and the protein extract fractionates differently than expected on the polyacrylamide gel.

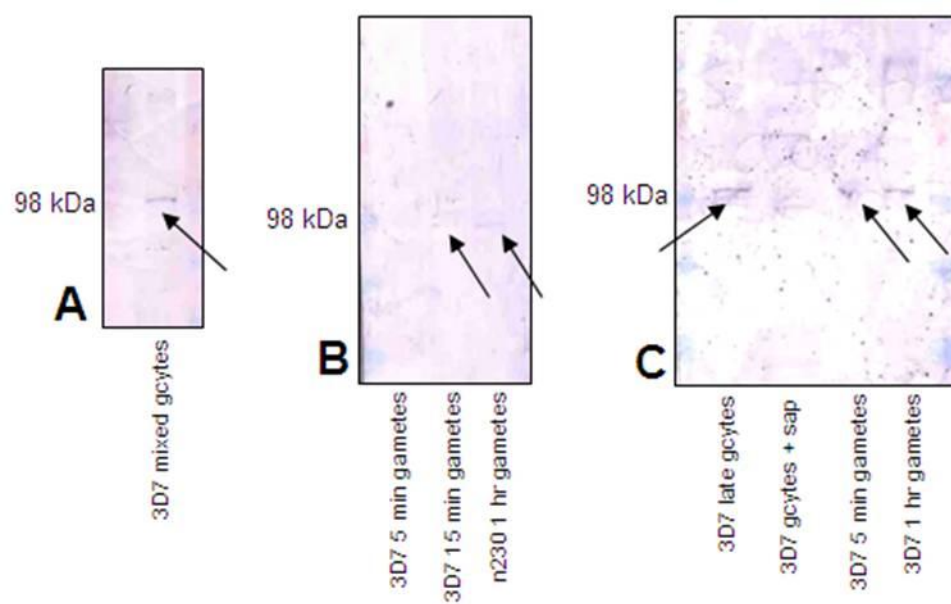


Figure 19. PFF0750w western blots of extracts from gametocytes and gametes. Three different western blots using 3D7 gametocyte and/or gamete extract are pictured (A, B, and C).

Since the mRNA expression profile of PFF0750w steadily increases throughout gametocytogenesis (Fig. 3) and there was little consistent reactivity in western blots with gametocyte extract, it was hypothesized that the protein itself is not actually expressed until the parasites become gametes. *P. falciparum* gametes were collected by inducing gametogenesis of stage V gametocytes by incubation with human serum and xanthurenic acid. After 5 minutes, 15 minutes, and/or 1 hour of gametogenesis, the gametes were collected using an Accudenz gradient, and proteins were extracted from the parasites using NETT. Late-stage gametocytes were often collected as well for these western blots, prior to gametogenesis induction. The extracts were size fractionated on 4-20% Tris-glycine polyacrylamide gels along with SeeBlue® standards, blotted on to nitrocellulose membranes which were stained with Ponceau S to confirm transfer (not pictured), and probed with the serum obtained from the mice that had been injected with the PFF0750w recombinant protein. The same reactive band at approximate size 98 kDa was again observed in gamete protein extract (Fig. 19B and C). However, once again, this band was faint and only became visible after a longer period of staining (>10 minutes). Also, reactive bands were not always observed in gamete protein extracts in western blots. Overall, a reactive band at 98 kDa was observed in gamete extract in four western blots, and no band was observed in six western blots.

#### PFF0750w Immunofluorescence Assays

The antibodies generated in mice against the PFF0750w recombinant protein were next tested for their ability to recognize parasite-produced PFF0750w protein by IFA. These assays were also used to analyze what life cycle stages expressed the PFF0750w protein. *P. falciparum* gametocytes were purified from culture as described, fixed on

coverslips with 1% formaldehyde (0.005% glutaraldehyde was also added to the fixing solution on some occasions), and probed with the pooled antiserum.

In initial IFA experiments, parasites from many gametocyte stages were tested for reactivity with these antibodies. No reactivity of the PFF0750w antibodies was observed with early-stage gametocytes. As the RNA expression profile showed increased expression throughout gametocytogenesis, stage V gametocytes and induced gametes were collected and used for subsequent IFA analysis. No convincing reactivity and fluorescence was observed between the PFF0750w antibodies and stage V *P. falciparum* gametocytes (not pictured). Five IFA analyses were conducted with stage V gametocytes and no reactivity was observed in any of the IFA experiments. There appeared to be a very small amount of fluorescence on stage V gametocytes in some IFA experiments conducted, however the fluorescence was similar to the background fluorescence seen in the negative control, and thus the antibody reactivity was not concluded to be positive.

IFA experiments with PFF0750w antibodies and *P. falciparum* gametes produced varied results. Throughout the ten IFA experiments performed with gamete slides, four experiments showed some reactivity and fluorescence of gametes probed with PFF0750w antiserum, while six experiments gave results of little to no reactivity and fluorescence (Fig. 20). Despite using the exact same procedure for each IFA experiment, the IFA results varied on different days; within a given IFA experiment, all gametes possessed similar levels of fluorescence. In row A of Figure 20, the gamete (lower center of figure) appears to be fluorescing the same amount as the background signal from the cellular debris right above the gamete. In row B, there appears to be more reactivity of the PFF0750w antibodies with the gametes on the slide, but the amount of background



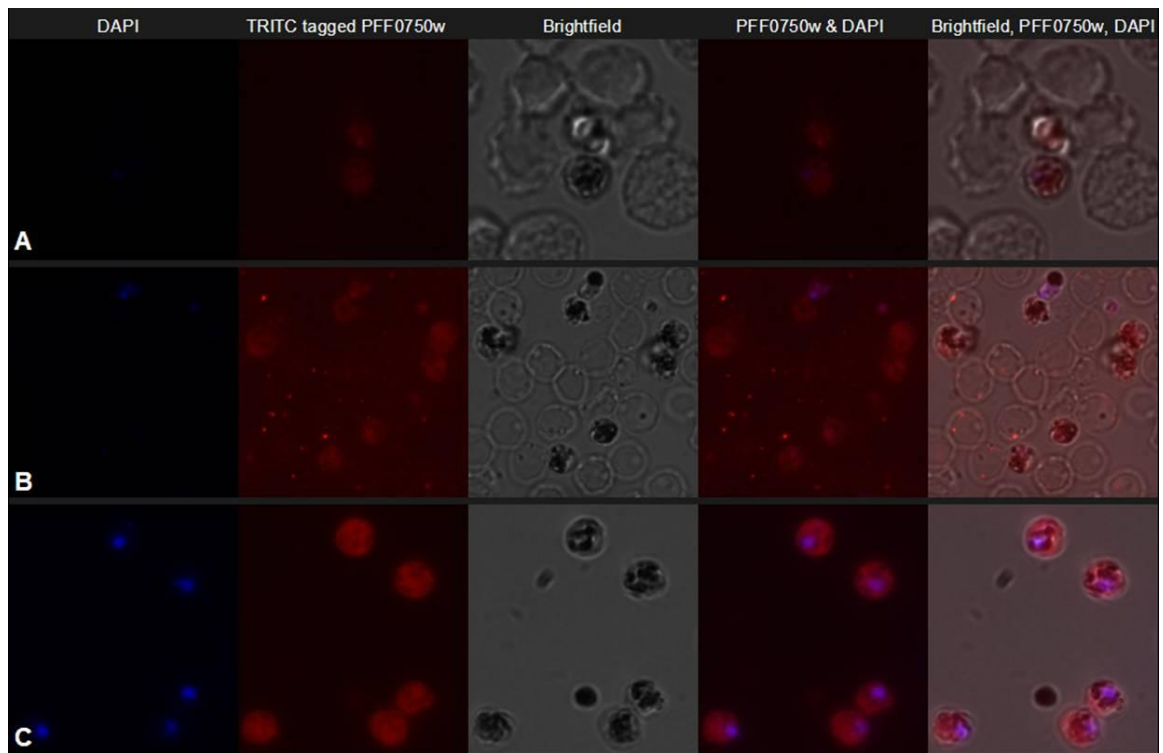


Figure 20. IFA analysis of PFF0750w protein expression in *P. falciparum* gametes.

fluorescence makes it difficult to be sure if the reactivity is specific and accurate. In row C, the gametes in this IFA experiment seemed to exhibit strong reactivity with the antiserum and high levels of fluorescence. However, this high fluorescence positive result was only obtained in a small percentage of the number of IFA experiments conducted. Similarly to the PFF0750w gamete western blot results, some degree of antibody reactivity was observed in only 50% of the gamete IFA experiments.

## CHAPTER IV

### DISCUSSION

The data in this study clearly indicate that PFL2550w protein is expressed primarily in early-stage gametocytes, is exported to the host RBC cytoplasm, and is localized in the RBC as a soluble protein. Our data also suggests that PFL2550w interacts with other export-related and RBC-localized *P. falciparum* proteins. The data supporting these findings will be discussed first and then the functional implications of this work will be addressed. With regard to our other target protein, PFF0750w, the data was less clear and new experimental approaches will be required to elucidate its subcellular location and function.

#### PFL2550w

##### *Generation of PFL2550w-specific antibodies*

First, PFL2550w-specific antibodies were generated in mice against a PFL2550w recombinant protein construct. The western blot of gametocyte extract provides support for the production of specific anti-PFL2550w antibodies. A distinct band was observed at 55.0 kDa in 3D7 gametocyte extract, which is consistent with the predicted 55.68 kDa size of the PFL2550w protein (Fig. 6). The slight size discrepancy can be attributed to the approximate nature of the method used to graph and interpolate the protein sizes. The 55.0 kDa band appears identical in intensity in the gametocyte extract lanes with and without BME, signifying that the protein is not coupled to another protein by disulfide

bonds and that the disruption of disulfide bonds does not significantly alter the size of the protein. There were no visible bands in the negative control RBC lanes, confirming the specificity of the antibodies to PFL2550w in a parasite protein extract. Additionally, there were no reactive bands in the asexual parasite lanes, suggesting that this protein is not expressed in the asexual parasite life cycle stages.

*Early gametocyte-specific expression of PFL2550w*

Western blot analysis also provided evidence that PFL2550w appears to be primarily expressed at high levels in gametocytes but expressed at lower levels in asexual parasites. Reactive bands at 55.0 kDa were observed in western blot analyses of Accudenz gradient purified gametocyte extracts (Fig. 6B, 7B, 7D, 8B). IFA analyses also provided evidence for PFL2550w expression in gametocytes. Figure 9 displays representative pictures of PFL2550w protein expression and localization through the life cycle. The general pattern of expression for each gametocyte stage was consistently observed across multiple IFAs and for most parasites at each stage within a given IFA analysis, strengthening our hypothesis that PFL2550w is gametocyte-specific.

PFL2550w was also expressed in a subpopulation of trophozoites that were committed to gametocytogenesis. This pattern of reactivity was most clearly seen in IFA experiments of parasites harvested at >6% parasitemia, 7-9 days after setting up an asexual culture at ~0.2%. These cultures included rings, trophozoites, schizonts, and stage II and III gametocytes. Conversely, PFL2550w was not found to be expressed consistently or in high levels in cultures with exclusively asexual parasites, harvested 4-5 days after setting up an asexual culture at ~0.2%. In IFA experiments with ring stage parasites, some PFL2550w protein expression was also observed in a subpopulation of

rings, though this expression was not consistently seen. In three IFA experiments with parasites from a low (<5%) parasitemia culture, a small subset of asexual rings exhibited some reactivity with the anti-PFL2550w antibodies. In the few rings in which reactivity was observed, the PFL2550w distribution was typically similar to that which is shown in Figure 9: there was some PFL2550w protein visualized on the RBC surface and some PFL2550w protein seen within the parasite itself. As PFL2550w protein expression was only observed in a subset of all parasites with asexual morphology, it is hypothesized that only parasites committed to sexual development and gametocytogenesis express the PFL2550w protein during the asexual cycle. Other sexual-specific *P. falciparum* proteins have been characterized and can be used as markers for sexual differentiation, including Pfs16, Pfs25, Pfg25/27, Pfs48/45, and Pfs230 (Dechering et al. 1997). Pfs16 is commonly used as a marker for sexual differentiation and commitment to gametocytogenesis. It is the first known sexual stage protein detected after the merozoite invasion of the erythrocyte and later is localized to the parasitophorous vacuole (Kongkasuriyachai et al. 2004). PFL2550w may also be a marker of sexual commitment in asexual parasites. Additional IFA analyses were conducted using parasites collected from low parasitemia cultures. No specific antibody reactivity was observed in trophozoite or ring stages, though faint reactivity was occasionally observed in schizonts.

Western blot data also suggests that PFL2550w is expressed at higher levels in early gametocytes (Fig. 8). No reactive bands were observed in the extracts of parasites harvested before the appearance of stage II parasites (Fig. 6B, 7D). In the timecourse experiment, a faint band is seen in the asexual parasite lane (Fig. 8B). Throughout this timecourse, Giemsa-stained smears of the cultures were made on each day to determine

the predominant stages of parasites in each sample. While the parasites collected in the “3D7 asexuals” sample at 14% parasitemia were primarily asexual stage parasites, some stage II gametocytes were also observed, indicating the presence of parasites committed to sexual differentiation. As the western blot from this sample was the only one in which a reactive band was observed in an asexual parasite lane, it seems likely that some of the trophozoite stage parasites in the culture were committed stage I gametocytes. Thus, the stage I and II gametocytes in the culture could have been responsible for the PFL2550w protein expression observed.

According to our western blot analysis, PFL2550w protein was expressed at the highest levels on timecourse days 10 and 12, which contain primarily stage I and II gametocytes and stage II and III gametocytes, respectively. Reactivity was lower on days 14 and 16, which contained primarily stage IV and V gametocytes, and there was no strong reactivity with gametes or asexual parasites. This data suggests that PFL2550w begins to be expressed during the induction of sexual differentiation, peaks in expression at stage I/II gametocytes, and then declines in expression. The IFA timecourse clearly demonstrated that PFL2550w is expressed principally in stage I, II, and III gametocytes and is not present in late stage gametocytes (Fig. 9). In stage I and II gametocytes, strong antibody reactivity was consistently observed, and PFL2550w was found to be distributed throughout the host RBC cytoplasm with very little, if any, protein localized to the parasite itself. Throughout its development, the parasite ingests and digests hemoglobin from its host RBC, altering the size and contents of the host cell (Mauritz et al. 2009). As the RBC shrinks in size and the parasite grows, PFL2550w protein appears to line the parasite’s exterior in RBCs infected with stage III gametocytes but remains localized to

the RBCs cytosol. In stage IV and V gametocytes and gametes, PFL2550w expression was not detected. The typical expression pattern of the PFL2550w protein throughout gametocytogenesis observed by IFA is very similar to the expression pattern observed by western blot and transcriptome analysis (Young et al. 2005). PFL2550w expression begins at the onset of sexual differentiation, peaks in stage I and II gametocytes, then declines, and by stage IV gametocytes, the protein is no longer present, suggesting a role in early gametocytogenesis.

*Localization of PFL2550w to the host erythrocyte cytosol*

The detection of PFL2550w in the cytosol of the RBC suggests that the protein must be exported from the parasite. As seen in stage I and II parasites (Fig. 9), strong antibody reactivity is observed throughout the RBC cytoplasm and little to no protein expression is seen in the parasite itself. Western blots also confirmed that PFL2550w protein is exported to the host RBC. A dark reactive band at 55.0 kDa was observed only in protein extract from parasites that were not treated with saponin (Fig. 7). Only a very faint band was seen in protein extract from parasites treated with saponin. Saponin treatment is known to permeabilize the infected RBC membrane as well as the parasitophorous vacuole (PV) membrane and the Mauer's clefts membranes (Spielmann et al. 2006). As saponin treatment permeabilizes the infected RBC membrane as well as the PV and Mauer's clefts membranes, the possibility that the PFL2550w protein resides in the PV or Mauer's clefts cannot be excluded by our western blot experiment alone. Streptolysin O (SLO) treatment, which permeabilizes the RBC membrane while keeping the PV and Mauer's clefts membranes intact, and subsequent analysis would be necessary to determine by western blot if the PFL2550w protein is found as a soluble protein in the

host RBC or is localized to the PV or Mauer's clefts (Spielmann et al. 2006). Using this information in conjunction with our results, we can conclude that PFL2550w does not localize to the PV or Mauer's clefts and most likely resides as a soluble protein in the cytosol of the RBC.

To further define the location of PFL2550w, additional IFA analyses were conducted comparing PFL2550w expression to previously characterized *P. falciparum* proteins. When a *P. falciparum* parasite enters a RBC, the parasite induces changes in the host cell including the formation of membrane-bound organelles, such as the flat lamellar membranes located beneath the erythrocyte plasma membrane called Mauer's clefts (Bonney and Ménard 2008). The Mauer's clefts appear to act as secretory organelles that concentrate exported parasite proteins for delivery to the host erythrocyte membrane (Maier et al. 2009). Some exported *P. falciparum* proteins are known to reside in the clefts, including skeleton binding protein 1 (SBP1), which has been shown to participate in transporting PfEMP1 to the red cell membrane (Maier et al. 2008). As previously reported in the literature, a punctate pattern localized to the periphery of the RBC was observed for SBP1 protein in our IFA experiment (not pictured). Conversely, PFL2550w was localized throughout the RBC cytoplasm and did not appear to be localized to the Mauer's clefts with SBP1. This further confirmed the hypothesis that PFL2550w protein is localized to the host cell cytoplasm as a soluble protein and is not bound to or associated with organelles.

The presence of PFL2550w in the RBC cytoplasm was also supported by colocalization IFA experiments using anti-PFL2550w and anti-Pfs16, which localizes to the parasitophorous vacuole (Kongkasuriyachai et al. 2004). The Pfs16 protein is



primarily observed to be lining the parasite, which is expected as the parasitophorous vacuole surrounds the parasite in the host erythrocyte. A comparison of the protein expression patterns of Pfs16 and PFL2550w demonstrate that the red PFL2550w in the erythrocyte surrounds the green Pfs16 in the parasite (Fig. 10). This indicates that PFL2550w is located almost exclusively in the host erythrocyte and Pfs16 is adjacent to the parasite. If these proteins localized to the same location, the combination of fluorescent red and green tagging would be observed as yellow color. Consistent with a lack of protein overlap, no yellow color was observed in the parasite or the RBC.

In the IFA experiments conducted with anti-PFL2550w and anti-Pfs16, not all gametocytes observed were positive for both proteins (Fig. 12). This may have been due to the technical limitations of IFA analysis, and in fact all gametocytes may express both proteins. Alternatively, this finding could have a more significant cellular meaning, such as a similar function of PFL2550w and Pfs16 and the occasionally exclusive expression of only one of these proteins. In a single IFA experiment, different parasites on the slide appeared to be labeled to different degrees, producing varying levels of fluorescent signal. Overall the numbers of brightly and faintly fluorescing parasites appeared to be similar for the two antibodies, even though the same parasites were not necessarily bright or faint for both antibodies. This is most likely due to random inconsistencies in antibody labeling. Yet the proportion of untagged parasites remained the same; this would suggest that the two genes are expressed at the same time. We cannot exclude that there may be a subpopulation of gametocytes expressing either PFL2550w or Pfs16. Another observation from this experiment was that the majority of the parasites reacting faintly with anti-PFL2550w appeared to be schizonts. Most brightly anti-PFL2550w-labeled

parasites were identified as gametocytes, consistent with our hypothesis of PFL2550w expression in early gametocytes

PFL2550w's characterization as a soluble protein in the RBC was further confirmed by the requirement for a modified IFA protocol. In the literature studies on most *P. falciparum* proteins, typical IFA protocols using acetone or formaldehyde fixative solution are sufficient for analysis. Initial IFA results in this study using these fixation methods with the anti-PFL2550w antibodies were inconclusive and inconsistent. In the 2006 study by Spielmann et al., it was determined that acetone or formaldehyde fixation did not prevent the solubilization and release of the host RBC cytoplasm, and thus was not sufficient for the study of soluble exported proteins. The soluble protein REX3, a ring stage-specific protein exported to the host RBC, was unable to be observed by IFA analysis without the addition of 0.005% glutaraldehyde to the formaldehyde fixative solution. The addition of glutaraldehyde allowed for clear reactivity of the REX3 antibodies with the REX3 protein in the erythrocytes. Following the initiative of Spielmann et al., the addition of glutaraldehyde to the fixative solution provided clear and consistent results for almost all subsequent IFAs with the anti-PFL2550w antibodies. This adjustment in IFA procedure further confirmed that PFL2550w is a soluble protein located in the host erythrocyte.

#### *Interaction of PFL2550w with export-related and RBC-localized proteins*

After finding that PFL2550w was a soluble protein, expressed primarily in early gametocytes, and localized to the host RBC, an immunoprecipitation experiment was conducted to detect proteins that directly interact with PFL2550w and confirm the specificity of the antibodies for PFL2550w. The results show that PFL2550w was

immunoprecipitated and that PFL2550w interacts with other export-related and RBC-localized *P. falciparum* proteins. The migration of the PFL2550w protein in a western blot indicated that the immunoreactive band was slightly larger than the expected protein size (55.68 kDa) and larger than what was observed in other western blots (55.0 kDa) (Fig. 13). Subsequent mass spectrometric analysis confirmed that PFL2550w was immunoprecipitated. The size difference could be attributed to the use of a different brand of gel, NuSep 10-well 4-20% gels [NuSep Ltd.; Frenchs Forest NSW, Australia], rather than the Invitrogen gels used in other experiments, resulting in a slightly different running pattern. Alternatively, the reduced amount of protein in the sample, just PFL2550w and a small amount of other proteins rather than all *P. falciparum* proteins, could have caused the different running distance and size difference. Nevertheless, it was apparent that PFL2550w was indeed collected in the immunoprecipitation elutions.

Mass spectrometric analysis identified thirteen *Plasmodium* specific genes/proteins, including PFL2550w, that were present in the anti-PFL2550w column elutions and not present in the control anti-MBP elutions (Table 1). It is necessary to use a negative control column for mass spectrometry analysis as there is often a high background signal of proteins, including proteins that bind to the common antibody domain, to the support resin, or to the microcentrifuge tube, as well as protein complexes that precipitate during the overnight lysate/resin incubation. Two proteins, PF11\_0175 and PFE0040c, were further investigated because of their predicted cellular locations and functions (PlasmoDB.org [updated 2010]). Their mRNA expression profiles were compared to that of PFL2550w to look for further similarities and connections between the proteins (Fig. 14) (Young et al. 2005). All three genes have similar expression

profiles in NF54 gametocytes, showing an expression peak at the onset of gametocytogenesis (NF54 day 1) and declining expression throughout subsequent gametocyte stages. There may indeed be similar cellular mechanisms and promoters that induce the transcription and translation of these genes during gametocytogenesis. In asexual parasites, genes involved in export to the erythrocyte show similar patterns of expression in the ring stage. In fact, the components of the RBC export complex were first targeted for further study because of their similar expression patterns in ring stage parasites. Additional studies are needed to more closely examine the set of genes with a similar expression profile to PFL2550w.

PFE0040c codes for the PfEMP2 protein, also known as MESA, which is expressed in trophozoites and schizonts and is known to localize to the infected RBC membrane skeleton (Maier et al. 2010). MESA is a large protein that is exported to the host erythrocyte and accumulates under the plasma membrane underneath knobs of infected erythrocytes (Saul et al. 1992). MESA is involved in the formation of these knob structures, which are implicated in the cytoadherence and sequestration of infected erythrocytes (Botha et al. 2007). Interestingly, both PFL2550w and MESA are identified as Type IV HSP40 proteins because of their DnaJ-like domains. This similarity and MESA's localization to the infected erythrocyte may result in some interaction between PFL2550w and MESA. As HSP40 proteins are possibly involved in chaperone activity, PFL2550w and MESA may both be components of a protein trafficking system or protein folding network.

PF11\_0175 codes for the HSP101 (heat shock protein 101) protein that was recently characterized as being a component of a novel protein export machine in *P.*

*falciparum* parasites, responsible for transporting parasite proteins from the parasitophorous vacuole to the erythrocyte cytoplasm (de Koning-Ward et al. 2009). The HSP101 amino acid sequence has an N-terminal endoplasmic reticulum (ER) signal sequence for export into the parasitophorous vacuole. HSP101 is part of the HSP100 family, which typically form hexameric ring-shaped complexes with a central pore through which proteins are translocated (de Koning-Ward et al. 2009). From their study, de Koning-Ward et al. hypothesized that HSP101 is likely responsible for unfolding and feeding denatured proteins through its central channel (2009). In another study, HSP101 was identified in pull-downs as associating with ER protein HSP70 and Plasmepsin V, an ER aspartic protease proposed to be responsible for recognizing the *Plasmodium* export element (PEXEL) and cleaving it at the correct site, serving as a “gatekeeper” for exported proteins (Russo et al. 2010). Interestingly, both HSP101 and MESA were identified in another study as components of the detergent-resistant membrane (DRM) proteome, proteins that may be involved in merozoite invasion of erythrocytes (Sanders et al. 2007). The subcellular location of PFL2550w could indicate that it is possibly transported through the novel protein export machinery. During this transportation, there may be interaction between PFL2550w and HSP101. However, prior to the results presented in this study, HSP101 expression had not been formally tested in gametocytes.

From our IFA data in early gametocytes (Fig. 15), HSP101 appears to be lining the exterior of the parasite, though not in a continuous line as has been observed for proteins like Pfs16. A similar punctate pattern was observed in asexual parasites for HSP101 and is consistent with a role in the *Plasmodium* protein export machine. Interestingly, the HSP101 protein appears to be localized to the interior of the parasite in

later stage gametocytes. However, the fluorescent signal is not as bright in these parasites and more work is needed to determine if HSP101 relocates to the cytosol of late stage gametocytes. It is clear that the HSP101 and PFL2550w proteins are not found in the same location in the parasitized RBC, as HSP101 localizes to the parasite while the PFL2550w protein is localized to the host cell cytoplasm.

#### *Potential PFL2550w function*

Many soluble exported *P. falciparum* proteins have been identified in previous studies, though only a small subset of these soluble proteins are known to be located in the host erythrocyte cytoplasm (Maier et al. 2010). Of these proteins, some, including glycophorin binding protein 130 (GBP130), PFB0920w, and PF13\_0073, are believed to play roles in the alterations of infected RBC rigidity (Maier et al. 2010). Infected erythrocytes are known to become rigid upon infection with *P. falciparum*, which is likely due to the export of parasite proteins that then cross-link with the RBC cytoskeleton (Maier et al. 2008). Histidine-rich protein II (HRP2) is another soluble exported protein also known to be localized to the RBC cytoplasm, as well as on the infected erythrocyte membrane; it is found in concentrated packets in the host cell cytoplasm and is believed to be an important factor in the detoxification of heme (Noedl et al. 2002). PFL2550w protein may also have a role in host cell modifications and changes in rigidity, however its DnaJ domain and its classification as a HSP40 protein point to a potentially more complex function.

In general, HSP40 proteins, in conjunction with HSP70 proteins, are believed to serve as co-chaperones by targeting protein substrates to HSP70 to be folded and to stabilize HSP70 in a substrate bound form (Botha et al. 2007). HSP40 proteins are

classified as such because they possess a DnaJ domain and are implicated in additional functions such as the assembly and disassembly of higher order proteins and the translocation of proteins across membranes (Maier et al. 2010). Many of the predicted *P. falciparum* HSP40 proteins contain the PEXEL/HT export motif and are believed to be exported to the host erythrocyte, which seems fitting given the parasite's need to establish processes required by the parasite in the host cell, such as protein folding and trafficking (Botha et al. 2007). Interestingly, no *P. falciparum* HSP70 proteins contain a PEXEL/HT motif and are not predicted to be exported to the host RBC; exported *P. falciparum* HSP40 proteins may in fact function by trafficking other exported parasite proteins to human HSP70 proteins for folding and assembly (Botha et al. 2007). As an HSP40 protein, the role of PFL2550w in the RBC cytoplasm may be that of a co-chaperone, responsible for folding and trafficking proteins.

In a study on exported *P. falciparum* proteins, including PFL2550w, it was found that some exported proteins most likely participate at specific points in a protein transport pathway through the RBC (Maier et al. 2008). One protein believed to be trafficked by these exported cytoplasmic proteins is PfEMP1, a well-studied *P. falciparum* protein known for its roles in virulence and cytoadherence; in this way, these exported trafficking proteins directly contribute to the virulence and success of the parasite (Bonney and Ménard 2008). PfEMP1 is an essential protein that functions in the adherence of infected erythrocytes to endothelium in a variety of tissues, and this adherence is responsible for the clinical complications of *P. falciparum* infection (Hayward et al. 1999). PFL2550w may be found to be an important protein for *P. falciparum* virulence if it could be shown

that the removal of the PFL2550w gene and protein inhibits the trafficking of PfEMP1 to the RBC surface.

PFL2550w may also have a more specified cellular role due to its classification as a Type IV HSP40 protein. Type IV HSP40 proteins are distinguished from other types of HSP40 proteins by their variations in the typically conserved HDP (His-Pro-Asp) motif in the J domain (Maier et al. 2008). Type IV HSP40 proteins have not yet been found to bind to polypeptide substrates and thus may not function as chaperones. Type IV HSP40 proteins appear to be primarily but not quite exclusively found in apicomplexa, with *P. falciparum* possessing the largest number of Type IV proteins among the organisms studied (Botha et al. 2007). Maier et al. hypothesize that Type IV HSP40 proteins, such as PFL2550w, may have specialized roles in recruiting HSP70 for protein folding and assembly (2008). Botha et al. suggest that Types II and III HSP40 proteins may interact with Type IV HSP40 proteins to aid in interactions with the erythrocyte cytoskeleton (2007). PFL2550w likely has a specialized role in exported protein interactions similar to those hypothesized here.

The PFL2550w protein's interactions with HSP101 and PfEMP2 (MESA) identified in our immunoprecipitation also point to a potential function in RBC cytoplasmic protein trafficking. PfEMP2 is exported to the host erythrocyte and must be transported through the erythrocyte to its location near the RBC plasma membrane underneath parasite-constructed knobs. PFL2550w may be responsible for some step in the transportation process of PfEMP2. As HSP101 is known to be located on the parasitophorous vacuole membrane, it is unlikely that PFL2550w interacts with HSP101



in the same way. It seems more plausible that HSP101 and PFL2550w interact briefly as PFL2550w passes through the parasite export machine to the RBC.

While the localization of PFL2550w has been characterized and the function of the protein has been theorized, PFL2550w knockout parasites may help to provide a better understanding of the cellular role of PFL2550w. To that end, we have already created a total gene knockout and a gene truncation construct for PFL2550w. If transfectant clones can be obtained, the phenotype of these parasites will then be studied.

As we propose that PFL2550w has some function in protein trafficking in the erythrocyte, the removal of PFL2550w protein from the parasite may disrupt a trafficking system and prevent other proteins from reaching their correct destinations in the host RBC. Another possible phenotype is that the PFL2550w knockouts will not be able to produce the same quality or quantity of gametocytes as normal parasites, as PFL2550w mRNA was found to be expressed at a much higher level in a gametocyte-producing strain compared to a gametocyte deficient strain (unpublished data Eksi et al.). The numbers of mature gametocytes may be significantly reduced when the PFL2550w gene is removed; alternatively, the morphology of the knockout parasites may be changed in such a way that they are not as easily able to mature and develop. For example, *P. falciparum* parasites with disrupted gene Pfg27 were able to undergo all stages of gametocytogenesis and maturation into gametes, however a significant percentage of Pfg27-deficient gametocytes were found to have distinct abnormalities in their intra- and extra-cellular membranous compartments (Olivieri et al. 2009). Another possibility is that PFL2550w may have no obvious morphological alterations or other phenotype changes; the PFL2550w protein may not be essential for *in vitro* gametocyte development and

maturation. A thorough analysis of PFL2550 knockouts may likely reveal the true function and role of this gene in gametocytogenesis.

#### *PFL2550w summary*

This study has characterized PFL2550w as a soluble exported *P. falciparum* gametocyte protein localized to the host erythrocyte. The protein's location in the host RBC cytoplasm and its classification as a HSP40 protein suggest a role in the trafficking of other exported parasite proteins through the host erythrocyte. The classification of PFL2550w specifically as a Type IV HSP40 protein, due to its variation in the HPD motif of the J domain, implies that the protein may not have a specific role as a chaperone, but it seems likely that PFL2550w is involved in a trafficking system in the host RBC. The cloning and phenotype analysis of PFL2550w knockout parasites in culture will provide further clues to the cellular function of PFL2550w *in vivo* and will help determine if PFL2550w is necessary for parasite survival and virulence. The continued study of PFL2550w function in the host erythrocyte cytoplasm will reveal the true significance of this protein in the *P. falciparum* life cycle.

#### PFF0750w

##### *Generation of PFF0750w-specific antibodies*

PFF0750w, a putative cyclin-dependant kinase, was also included in this study. However, the results and findings in the study of this gene and protein were varied and inconclusive. The antibodies generated against PFF0750w recombinant protein may not be specific to PFF0750w. Though inconclusive, our data suggests that PFF0750w protein is expressed in late gametocytes and gametes. PFF0750w requires additional new experimental approaches to determine its location and function.

Similarly to the study on PFL2550w, PFF0750w recombinant protein was expressed in *E. coli*, purified, and injected into mice for antibody production. However, it is unclear from our experiments if the generated antibodies are specific to PFF0750w protein. The western blot data suggests that PFF0750w protein is expressed in stage V gametocytes and gametes, but this finding was not consistent across all experiments. The difficulties in observing PFF0750w expression in western blot and IFA experiments may be due to non-specific antibodies, but could also result from very low levels of PFF0750w protein in the parasite that were able to escape detection. Additionally, our many failed attempts at limited dilution cloning of the PFF0750w gene truncation constructs could suggest that PFF0750w protein is vital for parasite survival in the absence of other parasites.

First, the western blot and IFA analyses of the anti-PFF0750w antibodies suggest that the generated antibodies may not be specific to PFF0750w. The western blot experiments of stage V gametocytes and gametes occasionally revealed a reactive band at approximately 98 kDa, though this was not seen in all blots, despite using an identical protocol each time (Fig. 19). This occasionally observed band was much larger than the expected size of the PFF0750w protein, 83 kDa, so it is unclear if the antibodies were identifying the correct protein on the limited number of occasions when reactivity was seen. The antibodies could be recognizing another *P. falciparum* kinase with a similar amino acid sequence, such as mitogen-activated protein kinases 1, PF14\_0294 (107.3 kDa), or protein kinase 1, PF08\_0044 (104.6 kDa) [PlasmoDB.org]. However, it is possible that the *in vivo* PFF0750w protein may be larger than its predicted size due to posttranslational modifications such as phosphorylation or glycosylation. The IFA results

obtained using the collected antiserum were equally inconclusive. No IFA antibody reactivity was observed in any stage of asexual parasites or gametocytes. Some IFA analyses seemed to reveal specificity and reactivity of the antiserum to *P. falciparum* gametes, while many other gamete analyses revealed little to no reactivity (Fig. 20). It is not clear why this great discrepancy was seen in IFAs conducted using the exact same protocol, and it is unclear which level of reactivity is the true result for this antiserum.

These varied western blot and IFA results may have been caused by incorrect or nonspecific antibodies generated in the injected mice. When analyzing and verifying the PFF0750w recombinant protein elutions by polyacrylamide gel, it appeared that the majority of the recombinant protein was in a degraded state with a lower than expected molecular weight (Fig. 18). If the protein was too degraded at that time it was injected into mice, the antibodies generated in mice may have not been specific enough to the PFF0750w protein to be able to properly identify it in western blots or IFAs. If this is the case, recombinant PFF0750w protein needs to be remade and injected into new mice for antibody production. Another possibility is that the *E. coli*-produced protein may be in a different configuration and structure than the parasite-produced protein. Alternatively, if enough of the recombinant protein remained intact and undegraded at the time of injection into mice, the generated antibodies may have been correct and specific to PFF0750w. The problem then may be that there is such a small amount of PFF0750w protein present in *P. falciparum* parasites that it is unable to be detected by the methods used in this study. If the reactivity problems are due to a different protein structure of the *E. coli*-produced protein or very small amounts of PFF0750w protein *in vivo*, it may be

beneficial to tag PFF0750w with a GFP or HA tag, though these methods may also not be sensitive enough for accurate analysis.

#### *Targeted PFF0750w gene disruption*

A parasite line with a disrupted PFF0750w gene was successfully produced, but multiple attempts to isolate a clonal population of PFF0750w-disruptants have been unsuccessful thus far. Drug resistant parasites were obtained from two independent transfections of the PFF0750w truncation construct and named J1 and J2. When these cultures were analyzed by integration PCR, it was found that some parasites in both J1 and J2 had correctly integrated the gene truncation construct into their genomes; however, WT parasites and episomal DNA were also present in both cultures (Fig. 17). Limited dilution cloning has been attempted multiple times to isolate a population of parasites derived from one parasite with correct plasmid integration. Thus far, all cloning attempts have been unsuccessful. Cloning attempts may have failed because the PFF0750w gene and protein may be very important or even essential for normal parasite growth and development, and the disruption of this gene may result in parasite death. The PFF0750w truncation parasites may be able to survive in a mixed culture with WT parasites by utilizing the WT PFF0750w protein, but then may die when isolated from WT parasites. Continued cloning attempts will be conducted to isolate parasites with correct PFF0750w gene truncation construct integration. If clonal parasites can be isolated, the phenotype will be studied to elucidate the cellular role of PFF0750w. As a putative cyclin-dependant kinase and a cdc2-like kinase, it is possible that PFF0750w does play a role in cell cycle regulation and may possibly prevent parasites from replicating and dividing normally through their cell cycle.

*PFF0750w summary*

More study is needed to determine the function and localization of the PFF0750w protein. The similar expression profiles of PFF0750w and Pfgdv1, found to be deleted in a strain of gametocyte deficient parasites, as well as PFF0750w's classification as a putative cyclin-dependant kinase, point to potentially significant cellular functions of PFF0750w. Further study is needed to determine if disrupting the PFF0750w gene creates a block in the parasite's natural cell cycle or disrupts the parasites' normal development and replication in some way. PFF0750w may prove to be a vital gene for *P. falciparum* survival and may be a target in the future for anti-malarial drug development.

## REFERENCES

- Alano P. 2007. *Plasmodium falciparum* gametocytes: still many secrets of a hidden life. *Mol Microbiol* 66(2):291-302.
- BLAST: Basic Local Alignment Search Tool. NCBI. [Internet] [updated 2010]. Available from: <http://blast.ncbi.nlm.nih.gov/Blast.cgi>.
- Blisnick T, Morales Betoulle ME, Barale JC, Uzureau P, Berry L, Desroses S, Fujioka H, Mattei D, Braun Benton C. 2000. *Pfsbp1*, a Maurer's cleft *Plasmodium falciparum* protein, is associated with the erythrocyte skeleton. *Mol Biochem Parasitol* 111(1):107-121.
- Bonnefoy S, Ménard R. 2008. Deconstructing Export of Malaria Proteins. *Cell* 134:20-22.
- Botha M, Pesce ER, Blatch GL. 2007. The Hsp40 proteins of *Plasmodium falciparum* and other apicomplexa: Regulating chaperone power in the parasite and the host. *Int J Biochem Cell B* 39:1781-1803.
- Bruce MC, Alano P, Duthie S, Carter R. 1990. Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* 100:191-200.
- Caplan AJ, Cyr DM, Douglas MG. 1993. Eukaryotic homologues of *Escherichia coli* dnaJ: A diverse protein family that functions with HSP70 stress proteins. *Mol Biol Cell* 4:555-563.
- CDC: Center for Disease Control and Prevention; Malaria. [Internet] [updated 2006 Sept 21]. Available from: <http://www.cdc.gov/malaria/disease.htm>.
- Cheetham ME, Caplan AJ. 1998. Structure, function and evolution of DnaJ: conservation and adaptation of chaperone function. *Cell Stress Chaperon* 3(1):28-36.
- Craig A, Scherf A. 2001. Molecules on the surface of the *Plasmodium falciparum* infected erythrocyte and their role in malaria pathogenesis and immune evasion. *Mol Biochem Parasitol* 115:129-143.

- de Koning-Ward TF, Gilson PR, Boddey JA, Rug M, Smith BJ, Papenfuss AT, Sanders PR, Lundie RJ, Maier AG, Cowman AF, Crabb BS. 2009. A newly discovered protein export machine in malaria parasites. *Nature* 459:945-950.
- Dechering KJ, Thompson J, Dodemont HJ, Eling W, Konings RNH. 1997. Developmentally regulated expression of *pfs16*, a marker for sexual differentiation of the human malaria parasite *Plasmodium falciparum*. *Mol Biochem Parasitol* 89(2):235-244.
- Doerig C, Doerig C, Horrocks P, Coyle J, Carlton J, Sultan A, Arnot D, Carter R. 1995. Pfcrk-1, a developmentally regulated cdc2-related protein kinase of *Plasmodium falciparum*. *Mol Biochem Parasitol* 70:167-174.
- Eksi S, Haile Y, Furuya T, Ma L, Su X, Williamson KC. 2005. Identification of a subtelomeric gene family expressed during the asexual-sexual stage transition in *Plasmodium falciparum*. *Mol Biochem Parasitol* 143(1):90-99.
- Epp C, Deitsch K. 2006. Deciphering the export pathway of malaria surface proteins. *Trends Parasitol* 22(9):401-404.
- Geyer JA, Keenan SM, Woodard CL, Thompson PA, Gerena L, Nichols DA, Gutteridge CE, Waters NC. 2009. Selective inhibition of Pfmrk, a *Plasmodium falciparum* CDK, by antimalarial 1,3-diaryl-2-propenones. *Bioorg Med Chem Lett* 19(7):1982-1985.
- Geyer JA, Prigge ST, Waters NC. 2005. Targeting malaria with specific CDK inhibitors. *BBA-Proteins Proteom* 1754(1-2):160-170.
- Goodman AL, Draper SJ. 2010. Blood stage malaria vaccines – recent progress and future challenges. *Ann Trop Med Parasitol* 104(3):189-211.
- Greenwood BM, Fidock DA, Kyle DE, Kappe SHI, Alonso PL, Collins FH, Duffy PE. 2008. Malaria: progress, perils, and prospects for eradication. *J Clin Invest* 118(4):1266-1276.
- Hayward RE, Tiwari B, Piper KP, Baruch DI, Day KP. 1999. Virulence and transmission success of the malarial parasite *Plasmodium falciparum*. *P Natl Acad Sci USA* 96:4563-4568.
- Jones TR, Hoffman SL. 1994. Malaria Vaccine Development. *Clin Microbiol Rev* 7(3):303-310.
- Kappes B, Doerig CD, Graeser R. 1999. An overview of *Plasmodium* protein kinases. *Parasitol Today* 15(11):449-454.



- Kelly-Hope KA, McKenzie FE. 2009. The multiplicity of malaria transmission: a review of entomological inoculation rate measurements and methods across sub-Saharan Africa. *Malar J* 8(19).
- Kinnaird JH, Mottram JC. 1997. *Plasmodium* cdc2-related kinases: do they regulate stage differentiation? *Parasitol Today* 13(1):7-8.
- Kongkasuriyachai D, Fujioka H, Kumar N. 2004. Functional analysis of *Plasmodium falciparum* parasitophorous vacuole membrane protein (Pfs16) during gametocytogenesis and gametogenesis by targeted gene disruption. *Mol Biochem Parasitol* 133(2):275-285.
- Maier AG, Braks JAM, Waters AP, Cowman AF. 2006. Negative selection using yeast cytosine deaminase/uracil phosphoribosyl transferase in *Plasmodium falciparum* for targeted gene deletion by double crossover recombination. *Mol Biochem Parasitol* 150:118-121.
- Maier AG, Cooke BM, Cowman AF, Tilley L. 2009. Malaria parasite proteins that remodel the host erythrocyte. *Nat Rev Microbiol* 7(5):341-354.
- Maier AG, Rug M, O'Neill MT, Brown M, Chakravorty S, Szeszak T, Chesson J, Wu Y, Hughes K, Coppel RL, Newbold C, Beeson JG, Craig A, Crabb BS, Cowman AF. 2008. Exported proteins required for virulence and rigidity of *Plasmodium falciparum*-infected human erythrocytes. *Cell* 134:48-61.
- Mauritz JMA, Esposito A, Ginsburg H, Kaminski CF, Tiffert T, Lew VL. 2009. The homeostasis of *Plasmodium falciparum*-infected red blood cells. *PLOS Comput Biol* 5(4): e1000339.
- Merckx A, Le Roch K, Nivez M, Dorin D, Alano P, Gutierrez GJ, Nebreda AR, Goldring D, Whittle C, Patterson S, Chakrabarti D, Doerig C. 2003. Identification and initial characterization of three novel cyclin-related proteins of the human malaria parasite *Plasmodium falciparum*. *J Biol Chem* 278(41):39839-39850.
- Noedl H, Wernsdorfer WH, Miller RS, Wongsrichanalai C. 2002. Histidine-Rich Protein II: a Novel Approach to Malaria Drug Sensitivity Testing. *Antimicrob Agents Chemother* 46(6): 1658-1664.
- Olivieri A, Camarda G, Bertuccini L, van de Vegte-Bolmer M, Luty AJF, Sauerwein R, Alano P. 2009. The *Plasmodium falciparum* protein Pfg27 is dispensable for gametocyte and gamete production, but contributes to cell integrity during gametocytogenesis. *Mol Microbiol* 73(2):180-193.
- Pelech SL, Sanghera JS, Daya-Makin M. 1990. Protein kinase cascades in meiotic and mitotic cell cycle control. *Biochem Cell Biol* 68(12):1297-1330.

- PlasmoDB: The Plasmodium Genome Resource. [Internet] [updated 2010 Jan 5] Version 6.3. Available from: <http://plasmodb.org/plasmo/>.
- Russo I, Babbitt S, Muralidharan V, Butler T, Oksman A, Goldberg DE. 2010. Plasmeprin V licenses *Plasmodium* proteins for export into the host erythrocyte. *Nature* 463(7281):632-636.
- Sanders PR, Cantin GT, Greenbaum DC, Gilson PR, Nebl T, Moritz RL, Yates JR, Hodder AN, Crabb BS. 2007. Identification of protein complexes in detergent-resistant membranes of *Plasmodium falciparum* schizonts. *Mol Biochem Parasitol* 154(2):148-157.
- Sargeant TJ, Marti M, Caler E, Carlton JM, Simpson K, Speed TP, Cowman AF. 2006. Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. *Genome Biol* 7(2):R12.
- Saul A, Yeganeh F, Howard RJ. 1992. Conservation of repeating structures in the PfEMP2/MESA protein of *Plasmodium falciparum*. *Immunol Cell Bio* 70:353-355.
- Silvestrini F, Bozdech Z, Lanfrancotti A, Di Giulio E, Bultrini E, Picci L, deRisi JL, Pizzi E, Alano P. 2005. Genome-wide identification of genes upregulated at the onset of gametocytogenesis in *Plasmodium falciparum*. *Mol Biochem Parasitol* 143:100-110.
- Spielmann T, Hawthorne PL, Dixon MWA, Hannemann M, Klotz K, Kemp DJ, Klonis N, Tilley L, Trenholme KR, Gardiner DL. 2006. A cluster of ring stage-specific genes linked to a locus implicated in cytoadherence in *Plasmodium falciparum* codes for PEXEL-negative and PEXEL-positive proteins exported into the host cell. *Mol Biol Cell* 17:3613-3624.
- Striepen B, Jordan CN, Reiff S, Dooren. 2007. Building the perfect parasite: cell division in Apicomplexa. *PLOS Pathog* 3(6):691-698.
- Talman AM, Domarle O, McKenzie FE, Arieu F, Robert V. 2004. Gametocytogenesis: the puberty of *Plasmodium falciparum*. *Malar J* 3:24.
- Walsh P, Bursac D, Law YC, Cyr D, Lithgow T. 2004. The J-protein family: modulating protein assembly, disassembly and translocation. *EMBO rep* 5(6):567-571.
- Ward P, Equinet L, Packer J, Doerig C. 2004. Protein kinases of the human malaria parasite *Plasmodium falciparum*: the kinome of a divergent eukaryote. *BMC Genomics* 5:79.

- Watanabe J. 1997. Cloning and characterization of heat shock protein DnaJ homologues from *Plasmodium falciparum* and comparison with ring infected erythrocyte surface antigen. *Mol Biochem Parasit* 88:253-258.
- White NJ, Pongtavornpinyo W, Maude RJ, Saralamba S, Aguas R, Stepniewska K, Lee SJ, Dondorp AM, White LJ, Day NPJ. 2009. Hyperparasitaemia and low dosing are an important source of anti-malarial drug resistance. *Malar J* 8:253.
- WHO: World Health Organization. [Internet] [updated 2009 Jan]. Available from: <http://www.who.int/topics/malaria/en/>.
- Williamson KC. 2003. Pfs230: from malaria transmission-blocking vaccine candidate toward function. *Parasite Immunol* 25:351-359.
- Williamson KC, Keister DB, Muratova O, Kaslow DC. 1995. Recombinant Pfs230, a *Plasmodium falciparum* gametocyte protein, induces antisera that reduce the infectivity of *Plasmodium falciparum* to mosquitoes. *Mol Biochem Parasitol* 75(1):33-42.
- Wirth DF. 2002. The parasite genome: Biological revelations. *Nature* 419:495-496.
- Wu Y, Kirkman LA, Wellems TE. 1996. Transformation of *Plasmodium falciparum* malaria parasites by homologous integration of plasmids that confer resistance to pyrimethamine. *P Natl Acad Sci USA* 93:1130-1134.
- Young JA, Fivelman QL, Blair PL, de la Vega P, Le Roch KG, Zhou Y, Carucci DJ, Baker DA, Winzeler EA. 2005. The *Plasmodium falciparum* sexual development transcriptome: A microarray analysis using ontology-based pattern identification. *Mol Biochem Parasitol* 143:67-79.

## **VITA**

Carolyn Jane (Bazzoli) Strobel was born in Geneva, Illinois and raised in Saint Charles, Illinois. In August of 2004, Carolyn started her undergraduate career at Grove City College in Grove City, Pennsylvania. She graduated Summa Cum Laude from Grove City College in May 2008 with a B.S. in Biochemistry and a second major in Christian Thought. Carolyn first came to Loyola University Chicago in the summer of 2006 when she participated in the Bioinformatics Research Experience for Undergraduates (REU) program and performed research in Dr. Kim Williamson's malaria lab. In the summer of 2007, Carolyn participated in a second REU program at Academia Sinica in Taipei, Taiwan, where she did biochemistry and biophysics research in Dr. Rita P.-Y. Chen's prion lab in the Institute of Biological Chemistry. In August of 2008, Carolyn entered the Department of Biology at Loyola University Chicago to pursue a Master of Science degree. She was awarded a two-year stipend and tuition scholarship by The Graduate School for 2008-2010.